PHENOTYPIC AND GENOTYPIC DIVERSITY OF *XANTHOMONAS AXONOPODIS* PV. *MANIHOTIS* CAUSING CASSAVA BACTERIAL BLIGHT DISEASE IN KENYA

MARY NJERI CHEGE

(BIOINFORMATICS AND MOLECULAR BIOLOGY)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

2018

Phenotypic and Genotypic Diversity of *Xanthomonas axonopodis* pv. *manihotis* Causing Cassava Bacterial Blight Disease in Kenya

Mary Njeri Chege

A Thesis Submitted in Partial Fulfillment for the Degree of Master of Science in Bioinformatics and Molecular Biology in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature:…….. Date:……

Mary Njeri Chege

This thesis has been submitted for examination with our approval as University Supervisors:

Signature:…….. Date:……

Prof. Fred Wamunyokoli , PhD

JKUAT, Kenya

Signature:…….. Date:……

Dr. Evans N. Nyaboga , PhD

UoN, Kenya

Signature:…….. Date:……

Dr. Joseph Kamau , PhD

UoN, Kenya

DEDICATION

I dedicate this thesis to the Almighty God for it is with His blessings of life, wisdom, and ability to learn that this work was accomplished.

To my family, I lovingly dedicate this work to you. Your immense support has been the solid foundation upon which this work firmly stood and your encouragement, love and understanding the fuel, with which it has gone on to burn to completion. God bless you.

ACKNOWLEDGEMENT

I wish to thank the Almighty God for his protection throughout the period of the research work.

I wish also to express my sincere thanks to my supervisors: Prof. Fred Wamunyokoli, Department of Biochemistry, Jomo Kenyatta University of Agriculture and Technology, Drs. Evans Nyaboga and Joseph Kamau, Department of Biochemistry, University of Nairobi, for their invaluable comments, skillful supervision and materials contributions which enabled me to complete this research project.

My sincere thanks and appreciation to Margaret Chege for her support and encouragement, all my colleagues in the laboratory, classmates and technical staff in the Department of Biochemistry, University of Nairobi, for their kind cooperation and encouragement. I am greatly indebted to you all.

Lastly, I wish to express my sincere thanks to my brother and sisters, for their prayers and inspiration towards achieving this goal.

To God be the glory, honour and power forever more, Amen!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ACMV	African cassava mosaic virus
AFLP	Amplified fragment length polymorphism
Bp	Base pairs
CBB	Cassava bacterial blight
CBSD	Cassava brown streak disease
CFU	Colony forming unit
CIAT	International Centre for Tropical Agriculture
CMD	Cassava mosaic disease
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EACMV	East African cassava mosaic virus
EDTA	Ethylenediamine tetra-acetic acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistical Databases
HCL	Hydrochloric acid
IITA	International Institute of Tropical Agriculture
Ml	Milliliters
Mm	Millimolar
NaCl	Sodium chloride

PCA	Principal component analysis
PCR	Polymerase chain reaction
PSA	Proportion of shared alleles
Pv	Pathovar
RAPD	Random amplified polymorphic DNA
rDNA	Ribosomal deoxyribonucleic acid
REP	Repetitive extragenic palindromic elements
REP-PCR	Repetitive extragenic palindromic elements-polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SSA	Sub-Saharan Africa
SPSS	Statistical package for social sciences
TAE	Tris acetic acid
TAL	Transcriptional activator-like
TE	Tris ethylenediamine tetra-acetic acid
UPGMA	Unweighted pair group arithmetic mean
Xam	*Xanthomonas axonopodis* pv. *manihotis*
YPG	Yeast peptone glucose
YPGA	Yeast peptone glucose agar

YPGA-CC Yeast peptone glucose agar - Cephalexin and Cycloheximide

ABSTRACT

Cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) is a vascular disease that can cause up to 100% yield losses in cassava growing regions in Kenya. There is no information available on the phenotypic and genetic variability of *Xam* isolates from Kenya. The objective of the present study was to determine the diversity of *Xam* isolates from different cassava growing regions of Kenya using phenotypic characteristics and DNA polymerase chain reaction-based fingerprinting (rep-PCR) using repetitive extragenic palindromic (rep) primers. Thirty three leaf samples showing symptoms of cassava bacterial blight disease were collected from major cassava growing regions in Kenya. *Xam* was isolated from the leaf samples and subjected to morphological and molecular characterization. A total of 26 bacterial isolates which produced mucoidal colonies on semi-selective media were obtained from 33 samples collected from farmer's fields. All the 26 bacterial isolates produced mucoidal colonies on YPGA-CC selective media. Biochemical tests of the 26 bacterial isolates indicated that all the isolates produced Gram-negative mucoidal thread-like rods in 3% potassium hydroxide (KOH). A dendrogram generated from analysis of phenotypic characteristics of the isolates produced two major clusters at 75% similarity level. Analysis of 19 isolates with repetitive extragenic palindromic (rep) PCR yielded characteristic fingerprint pattern with bands ranging between 400 and 2000 bp in size and their numbers ranged from 1 to 6 bands per isolate. Cluster analysis using unweighted pair group method with arithmetic averages (UPGMA) did not reveal any significant differences in clustering and relationship to the geographical origin, with exception of a single isolate that had unique fingerprints. These findings indicate that *Xam* population in Kenya evolved from the same origin and is a uniform population, and this may prove useful in future breeding programmes. The high genetic homogeneity indicates that development and use of resistant varieties will effectively control the disease.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Cassava (*Manihot esculenta* Crantz) is an important food crop for over 800 million people worldwide (FAO, 2013; Liu *et al.*, 2011). It is currently the fourth most important source of carbohydrate after rice, sugarcane and sorghum in sub-Saharan Africa (SSA) (Owolade *et al*., 2006) and it is estimated that 280 million people in SSA derive half of their daily calories from cassava (FAO, 2013). The crop is drought tolerant and performs well in low fertility soils making it an attractive crop especially to small-scale farmers with limited resources. Cassava storage roots are used for animal feed, industrial starch production and income generation for many small-scale farmers (Kawano, 2003). The storage roots and leaves are available all year round (Ntawuruhunga *et al*., 2006), making cassava an important food security crop, especially in drought-prone areas (Chavez *et al*., 2005). In Kenya, cassava is grown in over 90,000 ha, with an annual production of about 540,000 tons. Cultivation of cassava is done mainly in Nyanza, and Western, Eastern and Coast provinces.

However, biotic constraints such as pests and diseases have played a major role in declining the yields of cassava. The effects of biotic factors are aggravated tenfold by suboptimal management practices. In East Africa, the current farmer yields are less than one fifth of the maximum yields recorded in the same region (Fermont *et al.*, 2009). The presence of a variety of diseases has greatly constrained the maximum production. The major diseases of cassava in sub-Saharan Africa include cassava bacterial blight (CBB) caused by *Xanthomonas axonopodis* pv. *Manihotis* (*Xam*), cassava mosaic disease (CMD) (Hillocks and Wydra, 2002; Wydra and Verdier, 2002) and cassava brown streak disease caused by whitefly transmitted Begomoviruses (family Geminiviridae) (Bull *et al*., 2003, 2006).

Cassava bacterial blight (CBB) caused by *Xam* is a vascular disease that reduces the production of cassava up to 100% across sub-Saharan Africa and South America since it affects both

planting material and yields (Verdier *et al*., 2004; Ogunjobi and Dixon, 2006). Diverse symptoms are displayed by CBB infected cassava plants including wilting of leaves, blighting, formation of angular leaf lesions and stem cankers, stem and leaf exudates production and dieback of stems. Infection of CBB commences with the multiplication of *Xam* in the leaves around stomata and eventually enters into the vascular system through the stomatal pores (Jorge *et al*., 2001). The entry of *Xam* into the vascular system triggers defense-response, which include suberin and lignin deposition, accumulation of phenolic compounds, and occlusion of vessels. The degree of the impact of CBB varies depending on the population of pathogens, their pathogenicity, and genotypes of cassava (Restrepo and Verdier, 2000).

The control measures for CBB include use of clean stem cuttings for propagation, crop rotation, and removal of infected plant material. However, none of these control measures has been found effective against CBB. These methods are aimed at reducing bacterial populations in the field (Lozano, 1986). As with most plant bacterial diseases, the use of resistant varieties would be the most effective method of managing CBB. Breeding of disease-resistant varieties through conventional breeding requires resistant donor parents. The development of cultivars with durable resistance to CBB, however, requires a detailed understanding of the diversity of pathogen populations. Variation of pathogen populations can relate to differences in pathogenicity and/or virulence of isolates of the same pathogen (McDonald and Linde, 2002), and such differences can be attributed to differences in genetic makeup of the bacterial isolates. Therefore, for effective control of CBB, the understanding of variation of pathogen populations in different regions is important for breeding because plants need to have resistance for all pathogen strains. In addition, to develop efficient disease management strategies, the genetic diversity of the pathogens population under the climatic conditions studied must be known because genetic diversity of *Xam* has been reported to be directly correlated with environmental conditions (Dixon *et al*., 2002). If the variation of the pathogen is not considered, supposedly resistant cultivars may become susceptible when planted in locations where strains that overcome this resistance develop and/or are already present (Ma *et al*., 2009).

Variability of pathogen populations can be determined using different methods including use of phenotypic and molecular markers. Phenotypic characterization is an important first step in the assessment of diversity in pathogenic bacteria; however it is time consuming and is highly error prone due to morphological plasticity, which is influenced by environmental conditions. Therefore, genotypic differences between strains can be combined with phenotypic analyses to augment pathogen diversity. A number of PCR based methods have been usedfor identification and genetic characterizationof *Xam* isolates including use of random amplified polymorphic DNA (RAPDs) (Miesfeld, 1999), restriction fragment length polymorphism (RFLPs) (verdier *et al*., 1994), amplified fragment length polymorphisms (AFLP) (Restrepo *et al*., 1999) and repetitive extragenic palindromic-polymerase chain reaction (rep-PCR). Repetitive extragenic palindromic PCR technique has been shown to be very useful for studying plant pathogen population structures (Vera Cruz *et al*., 1996). It is simple and fast and has been used for characterizing *xanthomonads* and *pseudomonads* (Louws *et al*., 1995; Vera Cruz *et al*., 1996). Rep-PCR is strongly recommended for genetic characterization of pathogenic bacteria because it is more precise in terms of identification and evaluation of diversity (Cubero *et al*., 2002).

To date, no information is available regarding the phenotypic and genetic variability of *Xam* populations causing CBB from cassava growing regions in Kenya. Therefore, the objectives of this study were to determine the diversity/variability of 26 isolates of *Xam* collected from different cassava production regions of Kenya.

1.2 Problem statement

Cassava is a food security crop and a source of income that provides livelihoods to over 300 million people in sub-Saharan Africa (FAO, 2013). One of the most destructive disease of this important food crop is cassava bacterial blight (CBB), caused by *Xam* (Ogunjobi *et al.*, 2010 and Castiblanco *et al*., 2013). The disease causes yield losses of over 100%, depending on the genotype and prevailing environmental conditions (Lozano 1986; Wydra and Verdier 2002), and in severe cases has contributed to situations of starvation in certain African countries (Ogunjobi and Dixon, 2006). Despite the importance of cassava as a food security crop in Africa, research

on *Xam* causing CBB disease has been neglected, as reflected in the lack of information on distribution and diversity of the pathogen in cassava growing regions of Kenya. In addition, over reliance of maize as the only major source of starch in Kenya has led to the crop not evolving from subsistence to a commercial crop that can be sought in the fight against food insecurity (Mutuku *et al*., 2013). Knowledge of the distribution, phenotypic and genetic diversity of *Xam* causing CBB will help develop control strategies and hence increase in cassava productivity.

1.3 Justification of the study

Bacteria blight of cassava is the most important disease of cassava worldwide due to its growing concern, widespread and destructive nature. Use of resistant cultivars is a simple, effective, safe and economical strategy for integrated management of major diseases in numerous crops. This strategy could be used to control CBB if resistant cultivars could be identified or developed. The development and deployment of cultivars with durable resistance to CBB, however, necessitates a detailed understanding of the diversity of pathogen populations. Variation of pathogen populations can relate to differences in pathogenicity and/or virulence of isolates of the same pathogen (McDonald and Linde, 2002), and such differences can be attributed to differences in genetic makeup of the bacterial isolates. Therefore, for effective control of CBB, understanding of the distribution and variation of pathogen populations in different regions is important for breeding because plants need to have resistance to all pathogen strains. In addition, genetic diversity of *Xam* has been reported to be directly related with environmental conditions (Dixon *et al*., 2002) and therefore there is need to determine diversity of the pathogen population from different climatic conditions. Information on the pathogen's diversity is a prerequisite for studying the epidemiology of the disease and, subsequently, for selection of disease resistance sources for crop breeding (Restrepo *et al*., 1997, 2000; Banito, 2003). Eighty percent of Kenya is marginal area and cassava being drought tolerant can enable the potential of these areas to be tapped thus helping to deal with the persistent food insecurity which is a common feature in these areas (Mwangomb'e *et al* 2018). The need to protect cassava against bacterial blight disease is therefore, a crucial aspect of enhancing the production of the crop. Therefore, the aim of this

study was to determine the phenotypic and genotypic variability of *Xam* isolates collected from different cassava growing regions of Kenya.

1.4 Null hypotheses

i. It is not possible to isolate *Xam* from leaf samples of CBB-infected cassava plants
ii. There is no phenotypic variation of *Xam* isolates from different geographic regions of Kenya.
iii. There is no genetic variation of *Xam* isolates from different geographic regions of Kenya

1.5 Objectives

1.5.1 General objective

To determine the phenotypic and genetic diversity of *Xam* isolates causing cassava bacterial blight disease in Kenya using phenotypic and molecular markers.

1.5.2 Specific objectives

The specific objectives were:

i. To isolate *Xam* isolates from infected cassava leaf samples collected different cassava growing regions in Kenya.
ii. To characterize *Xam* isolates from different cassava growing regions in Kenya using phenotypic and cultural characteristics.
iii. To determine genetic diversity of *Xam* isolates from different geographic regions of Kenya using molecular markers.

CHAPTER TWO

LITERATURE REVIEW

2.1 The botany of cassava

Cassava belongs to the plant family *Euphorbiaceae*, with several commercial species (Hershey, 2005). It is a dicotyledonous crop and belongs to genus *Manihot*; sub-species *Manihot esculenta* Crantz and species (*spp*.) *esculenta* (Allem *et al.*, 2002). It is the only species from the genus that is widely cultivated for human food and animal feed (Rogers and Appan, 1973; Onwueme, 1978; Mkumbira, 2002; Nassar, 2006). Cassava is a diploid with 36 chromosomes (Roca, 1984; Nassar, 2002), although it is believed to be a segmental allotetraploid because chromosomes at metaphase 1 and anaphase 1 show a high number of duplicated nucleolar chromosomes (Kawano, 1980). There are no genetic and cytological barriers in the species of the *Manihot* genus, thus there can be interspecific crosses between species within the genus (Nassar, 1994; 2002; 2007). Cassava cultivars have been classified according to morphological traits and cyanogenic glucoside content; however, this classification is not completely reliable since environmental factors influence the expression of these traits.

2.2 Economic importance of cassava

Cassava is an important root crop and source of calories and carbohydrate with nearly the highest starch content in tropical and sub-tropical regions of the world (Padmaja *et al*., 1994; Mkumbira, 2002; Nweke, 2004). It is estimated that 250 million people in sub-Saharan Africa (SSA) derive half of their daily calories from cassava (FAO, 2013). Cassava storage roots can remain in the soil for up to 3 years without spoilage providing a continuous food supply and is therefore an important food and income security crop (Nweke, 1994). About 70 % of the tuberous roots are used for human consumption either directly as food after cooking or in processed forms. The remaining 30% is used for animal feed and other industrial applications such as starch, glucose and alcohol production (Owiti, 2009). A typical diet based on cassava starch provides less than 30% of the minimum daily requirement for protein and only 10 - 20% of the required amounts of iron, zinc, vitamin A and vitamin E. Cassava storage roots are a dominant starch rich staples in

the diet of people in sub-Saharan Africa (Lancaster and Brooks, 1983). Cassava leaves therefore supplements the dietary requirements for proteins and vitamins as well as other minerals such as calcium and iron (Owiti, 2009).

Cassava is an important commodity in the industrial sector mainly because of its starch which is used as a raw material for various products. Cassava starch is used in the production of adhesives (Balagopalan, 2002). Glucose and dextrose are products of starch hydrolysis which are liquefied into sugar syrups, which are used as a substitute for sucrose, glucose and synthetic sweeteners (Balagopalan, 2002; Henry and Westby, 2000). The crop is also a major player in the bio-fuel industry, for example one tonne of cassava with a starch content of 30% has the capability of producing close to 280 litres of 96% pure ethanol (UNCTAD, 2009).

2.3 Cassava production

African countries contribute the largest share (approximately 56%) of global cassava production which stands at 268 million tons (FAO, 2014). Nigeria is the leading producer of cassava in Africa and in the world with its total production being a third more than Brazil's production. Other African countries that significantly contribute to the global cassava production include Democratic Republic of the Congo, Ghana, Madagascar, Mozambique, Tanzania, Kenya and Uganda. The expected production output for 2015 indicates a continued expansion of production in Africa, where cassava remains a strategic crop for both food security and poverty alleviation (FAO, 2014). Recent statistics indicate that worldwide production of cassava has increased in the past three decades from 118 million to 276 million tons with majority of the increase from small holder farms in sub Saharan Africa. According to FAO (2014), Nigeria had the highest production in Africa and the world at 47 million tons. Consumption per capita has been estimated at 80 kg per year in Africa, supplying an estimated 37% of all dietary energy (IITA, 2014).

Kenya has been experiencing declining yields per capita in agricultural produce. This has resulted in food shortages and the consequential high rates of food insecurity in the country. Cassava is seen as a high value traditional crop that has enormous industrial value. The Kenyan government and other interested organizations have tried to promote the crop but it has not evolved from

subsistence to a commercial crop that can be sought in the fight against food insecurity. It is mainly grown in the coastal and western parts of the country for commercial purposes; other regions in the country produce small quantities of the crop where it is grown alongside other main staple crops like maize (Mutuku *et al*., 2013). Cassava is grown on approximately 77,502 Ha with an annual production of 841,196 tonnes. Major factors that hinder the potential for maximum cassava production in Kenya include pests and diseases, unavailability of clean disease free planting material, poor soil fertility, poor cropping systems and lack of a functional value addition chain linking farmers to local and international markets**.**

2.4 Constraints to cassava production

Cassava production and utilization is threatened by several biotic and abiotic constraints. Diseases and pests are the greatest biotic stresses to cassava production across the east and central Africa sub-region (IITA, 2014). The main biotic constraints include pests (cassava hornworm, cassava mites, thrips and mealybugs) and a wide range of diseases caused by viruses, bacteria, fungi and nematodes. Cassava bacterial blight (CBB) is one of the major destructive diseases present in all areas where cassava is cultivated. The disease causes yield losses ranging from 50 to 100%, depending on the genotype and environmental conditions (Lozano 1986; Wydra and Verdier, 2002), and in severe cases has contributed to situations of starvation in certain African countries (Lozano 1975; Ogunjobi and Dixon 2006). Prevalence and severity of the disease have diminished in the last 10 years in areas of the world, such as South America, due to implementation of adequate cultural practices and the use of resistant cultivars. However, in other areas, such as Southeast Asia and sub-Saharan Africa, a considerable increase in the incidence of CBB has been observed, probably due to an increase in rainfall and temperature related to global climate change (Reynolds *et al*., 2015).

2.4.1 Cassava bacterial blight (CBB)

Cassava bacterial blight (CBB) is a vascular disease that causes huge losses of the crop in Africa (Lozano, 1975). In susceptible cultivars, CBB can cause up to 100% yield loss when environmental conditions optimum for disease outbreaks occur. CBB causes death of leaves and

stems and as such severe yield losses are often encountered when outbreaks of the disease occur. Severe outbreaks of CBB in epidemic proportions caused famine in Zaire (now Congo DR) and Nigeria in the early 1970s (Hahn and Williams, 1973; Terry, 1976). Severe outbreaks of the disease also results in shortage supply of planting materials (Lozano, 1986).

2.4.1.1 Causal agent, transmission and symptoms of CBB

The causal organism of CBB was first named *Bacillus manihotis* later *Phytomonas manihotis*. The name was changed to *Xanthomonas campestris* pv. *manihotis* and most recent nomenclature categorized *Xanthomonas campestris* pv. *manihotis* as a synonym of *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) (Ogunjobi *et al*., 2010). *Xam* is a Gram-negative slender rod growing on sucrose containing media producing non-pigmented colonies with a single polar flagellum. The bacterium enters the leaves through natural openings such as the stomata and hydathodes and multiplies in the intracellular spaces of the mesophyll and the vascular tissues of the plant. This in turn enables the pathogen to spread systematically and efficiently in the plant (Verdier *et al*., 2004). Stem infections have often been found to originate from punctures by the bug *Pseudotheraptusdevastans*, an occasional vector of the bacterium together with the grasshopper *Zonocerusvariegates* (López and Bernal, 2012).. Infection requires 12 hours of 90 - 100% relative humidity with temperatures of 22 – 26°C (Lozano, 1986).

Dispersal of *Xam* is mainly by plant water droplets mainly in windy regions (Lozano and Sequeira, 1974). Inoculum dispersal in larger fields is by exchange and planting of diseased vegetative planting materials (Boher and Verdier, 1994). The bacterium remains viable for many months in stems and gum, renewing activity in wet periods. The survival rate of the bacterium is higher in dry than in moist conditions and better in cassava debris than in rhizosphere soil of cassava weeds (Http://www.plantwise.org). Studies conducted by Thaveechai *et al*. (1993) also show that the pathogen can survive in the plant without symptoms in the dry season and symptoms will only be visible during the rainy season.

Distinctive symptoms of the disease include the appearance of water-soaked angular leaf spot or lesions on leaves of infected plants (Figure 1). The spots often start along the veins, margins and tips of leaf blades. As the disease develops, neighboring spots join to form large brown patches or blights killing the leaf blade as it expands (Figure 1). The leaf dries or wilts and finally falls. In some susceptible cultivars, creamy or yellowish brown gummy exudates are discharged on leaves or stems but often distinctively on leaf petioles of infected plants (Lozano and Sequeira, 1974). Petioles of blighted leaves are often horizontally orientated to the main stem axis. In advanced stages of the disease, dieback of stems is common. It is common also to find new shoots developing from dead ends of stems of severely infected plant (Lozano, 1986).

Figure 2.1: Symptoms of cassava bacterial blight (CBB) disease.

(A) Dark green to blue angular spots with a water soaked appearance; and (B) defoliation commences with the older leaves and progress upward along the stem (http://www.pictures of cassava bacterial blight).

2.4.1.2 Economic importance of CBB

Cassava bacterial blight is the most economically important bacterial disease of cassava with a worldwide distribution (Ogunjobi *et al.*, 2010). The main effect of CBB is the restriction of root development where severely infected plants do not develop storage roots, leading to loss of yields and income to farmers. Lozano (1989) performed field experiments in Brazil using a susceptible cassava cultivar and yield reductions of 72% (from an average of 28.9 tons/ha to 8.1 tons/ha) was observed in fully CBB-infested fields. One year after the first report of cassava bacterial blight disease in Nigeria, estimated yield losses were reported at 75% (http://www.plantwise.org). In some parts of Uganda two years after the disease was first observed, yield losses as high as 90 - 100% was reported (Otim *et al*., 1981). In Zaire, the epidemics between 1971 and 1973 in the Kasaï and Bandundu provinces led to severe starvation, because the importance of cassava roots and leaves as staple foods in these areas was greatly affected by this disease (http://www.plantwise.org). These studies confirm that cassava bacterial blight disease is an important biotic constraint to cassava production.

2.4.1.3 Management of cassava bacterial blight

There are various strategies that are used in the management of CBB. Losses can be greatly reduced by a combination of measures taken within the perspective of integrated pest management (IPM) (Lozano, 1986). In areas where cassava bacterial blight has not occurred, great care should be taken in the introduction of germplasm. Vegetative propagated material must be introduced as meristem culture multiplied *in vitro* and certified disease free (Vaunterin, 1995). The bacterium can be eliminated from true seed by heat treatment at 60 °C for 20 minutes, followed by drying in shallow layers at 30 °C overnight or at 50 °C for 4 hours and then planted in quarantine (Vaunterin, 1995). Early detection and destruction of plants showing symptoms of the disease can prevent or slow the spread of the disease in farmers' fields (Lozano, 1986).

Crop rotation and fallowing also helps manage CBB by taking advantage of the six months period that the bacterial survives in the soil (Vaunterin, 1995). Tools and equipment used to cut

planting materials aid in the infection of disease free cuttings and therefore, cutting tools should be regularly disinfected using a bactericide and by surface sterilizing in 10% bleach (George, 2002). In cases of sporadic occurrence of the disease, great care must be taken in collecting cuttings only from healthy plants and from the most lignified portion of the stem, up to 1 meter from the base, combined with visual inspection for the absence of vascular browning (http://www.plantwise.org; Lozano and Terry, 1977; Vaunterin, 1995). The disease is usually managed by the use of resistant cultivars and cultural practices (Boher andVerdier, 1994). The use of resistant cultivars has proven to be the most effective control measure for CBB.

However, cassava cultivars resistant to CBB are not often used by farmers, because they are not well adapted to particular agro-ecological zones, or may not have other desirable characteristics such as yield, flavor and dry matter content. The majority of commercial cassava cultivars today remain susceptible to CBB (López and Bernal, 2012). Therefore, there is need to breed high-yielding cassava cultivars resistant to different *Xam* strains and environmental conditions. This requires information on the diversity of *Xam* from different geographic regions.

2.5 Characterization of *Xam*

Xanthomonas axonopodis pv. *manihotis* strains from various region in the world have been characterized using phenotypic and molecular markers.

2.5.1 Use of phenotypic/cultural characterization

Xanthomonas are gram negative, aerobic, motile, rod shaped bacteria producing substances of varying composition depending on the source of carbon in the growth medium. They show a typical translucent, viscoid, smooth, mucoid growth on Yeast Peptone Glucose Agar (YPGA) media (Bradbury, 1984). Differences in phenotypic characteristics have also been reported by researchers to vary in terms of color, shape, contour (margin) and elevation among *Xanthomonas axonopodis* pv. *manihotis* strains (Rodriguez *et al*., 1987). Xanthomonas can also be characterized on the basis of carbon utilization where the fast-growing strains whose colonies are

big in size are known to utilize sucrose among a broad range of carbon sources like dextrose and glucose (Mehat *et al*., 2005).

2.5.2 Use of molecular markers

Molecular techniques have been reported to be useful in developing easy and quick methods of bacterial characterization which involve distinguishing genera, species and even strains. PCR-based methods, such as AFLP (Amplified Fragment Length Polymorphism), REP (Repetitive Element PCR) and ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus), have been tested for their discriminatory ability because they are easy, reproducible and provide a considerable amount of useful information on genetic diversity (Restrepo *et al*., 2000a).

Initial studies of the genetic diversity of *Xam* were concentrated in African populations. The first report, which included 49 strains collected in the 1970s and 1980s from Nigeria, Benin, Ivory Coast, Congo, Cameroon, Uganda, Kenya, Niger, Togo and Zaire, reported the absence of polymorphisms using RFLP probes against the rRNA region and very low levels of polymorphisms using a probe against a pathogenicity region (Verdier *et al*., 1993). The results suggested a clonal nature of the African populations. Higher levels of diversity have been detected in South American populations (Verdier *et al.,* 1994), the likely center of origin. More recent studies, which have concentrated on *Xam* populations in Nigeria and Togo, have described higher diversity (Verdier *et al*., 2004; Ogunjobi *et al*., 2010) with a higher number of haplotypes than previously reported. This reflects the fact that initial studies (Verdier *et al*., 1994b) did not differentiate haplotypes among *Xam* strains from different countries, whereas the recent studies can differentiate several haplotypes in comparable numbers of strains from a single country (Wydra and Verdier, 2002). While caution must be taken when comparing these studies since different molecular markers have been used in the more recent studies (AFLP and RAPDs), it is clear that *Xam* diversity has increased due to a continued increase in the evolvement of *Xam* population. It is possible that these new haplotypes have appeared with the introduction of new cassava cultivars in the field. This increase in bacterial diversity makes the deployment of

resistant cultivars an increasingly complex task and underscores the importance of continuous studies of diversity of bacterial populations in the field.

In contrast with the case in Africa, populations of *Xam* in South America are diverse, based on results from strains collected in the 1970s and 1980s (Onyeka *et al*., 2008). A notable set of studies on the diversity of *Xam* populations was performed by Restrepo and collaborators in the 1990s in Colombia, Venezuela and Brazil (Verdier *et al*., 1994a; Restrepo *et al*, 1996). Results from these studies indicated a high genetic diversity of the pathogen in South American populations mainly using RFLP from a plasmidic region containing the pthB gene (Restrepo and Verdier, 1997). In general, strains were not geographically differentiated, with some exceptions where a haplotype was characteristic of a specific edapho climatic zone (Restrepo and Verdier, 1997). Migration of these latter haplotypes could also be detected between different cassava growing zones in Colombia (Gonzalez *et al*., 2002). In addition, bacterial populations had the capacity to change from year to year (Gonzalez *et al*., 2002) such that peaks of diversity were obtained in times and locations where environmental conditions were conducive for disease development (Restrepo and Verdier, 1997). The diversity was also greater when fields were planted with a higher number of cassava varieties, suggesting host selection pressure (Restrepo *et al*., 2004). Higher levels of diversity have been found in sub-regions where cassava has been cultivated longer and also in regions where environmental conditions are conducive for development of the disease (Trujillo *et al*., 2014).

2.5.2.1 PCR- based fingerprinting or use of arbitrary primers

Several techniques such as Random Fragment Length Polymorphism (RFLP) analysis, genomic finger-printing, and rDNA analysis, have been used in the identification and characterization of bacterial strains and pathotypes. Families of short interspersed repetitive elements including repetitive extragenic palindromic (REP) elements, enterobacterial repetitive intergenic consensus (ERIC) sequences and the BOX element, have been used to generate genomic fingerprints of a variety of *Xanthomonas* isolates and identified pathovars and strains that were previously not distinguishable by other methods of classification (Hulton *et al*., 1991; Louws *et al.*, 1994; Lee *et*

al., 2008; Martin *et al.*, 1992). Among a variety of existing genotyping techniques, repetitive elements based -polymerase chain reaction (REP-PCR) provides high taxonomic resolution as well as acting as a rapid detector of diversity and evolution of the plant pathogen genome being studied (Busch *et al*., 1999, Olive *et al*., 1999 and Versalovic *et al.*, 1994).

REP-PCR is a genomic fingerprinting technique that generates specific strain patterns obtained by the amplification of repetitive DNA elements present along the bacterial genome (Busch *et al*., 1999; Olive *et al*., 1999; Lupski *et al*., 1992; Versalovic *et al*., 1991). Although REP is one of the several repetitive methods (REP/ERIC/BOX), it has been employed and suggested for independent use (Snelling *et al*., 1996). REP has proved more discriminatory than 16S rRNA PCR methods and restriction fragment length polymorphism (Sander *et al*., 1998; Abd-El-Haleem *et al*., 2002), and provides high discriminatory power (Patton *et al*., 2001). In addition, REP procedure is simpler than other genomic DNA protocols, such as pulsed field gel electrophoresis, for molecular typing (Sander *et al*., 1998). Rep-PCRs have been used to assess variation among pathovars of *Xanthomonas* species and have revealed low levels of intra-pathovar diversity (Cubero *et al*., 2002). No study has been done to determine the genetic variability of *Xam* populations from cassava growing regions in Kenya using rep-PCR.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of CBB- infected cassava samples from farmers' fields

Leaf samples were collected from diseased cassava plants showing bacterial blight symptoms from five cassava growing regions (Western, Coastal, Nyanza, Central and Eastern) of Kenya (Figure 3). A total of 33 cassava leaf samples with CBB disease symptoms were used in the study. Out of these, 7 samples from Western region were obtained from a previous ongoing study at Department of Biochemistry, University of Nairobi, while the other 26 samples were randomly collected from farmers' fields in Western and Coast which are the main cassava growing regions followed by Nyanza, Eastern and Central regions (Table 1.3). In addition, the locations where samples were collected were marked by recording the altitude, latitude and longitude using the Global Positioning System (GPS). The samples were collected in sterile khaki bags containing silica gel, transferred to the laboratory at the Department of Biochemistry, University of Nairobi and stored at room temperature for subsequent *Xam* isolation and characterization.

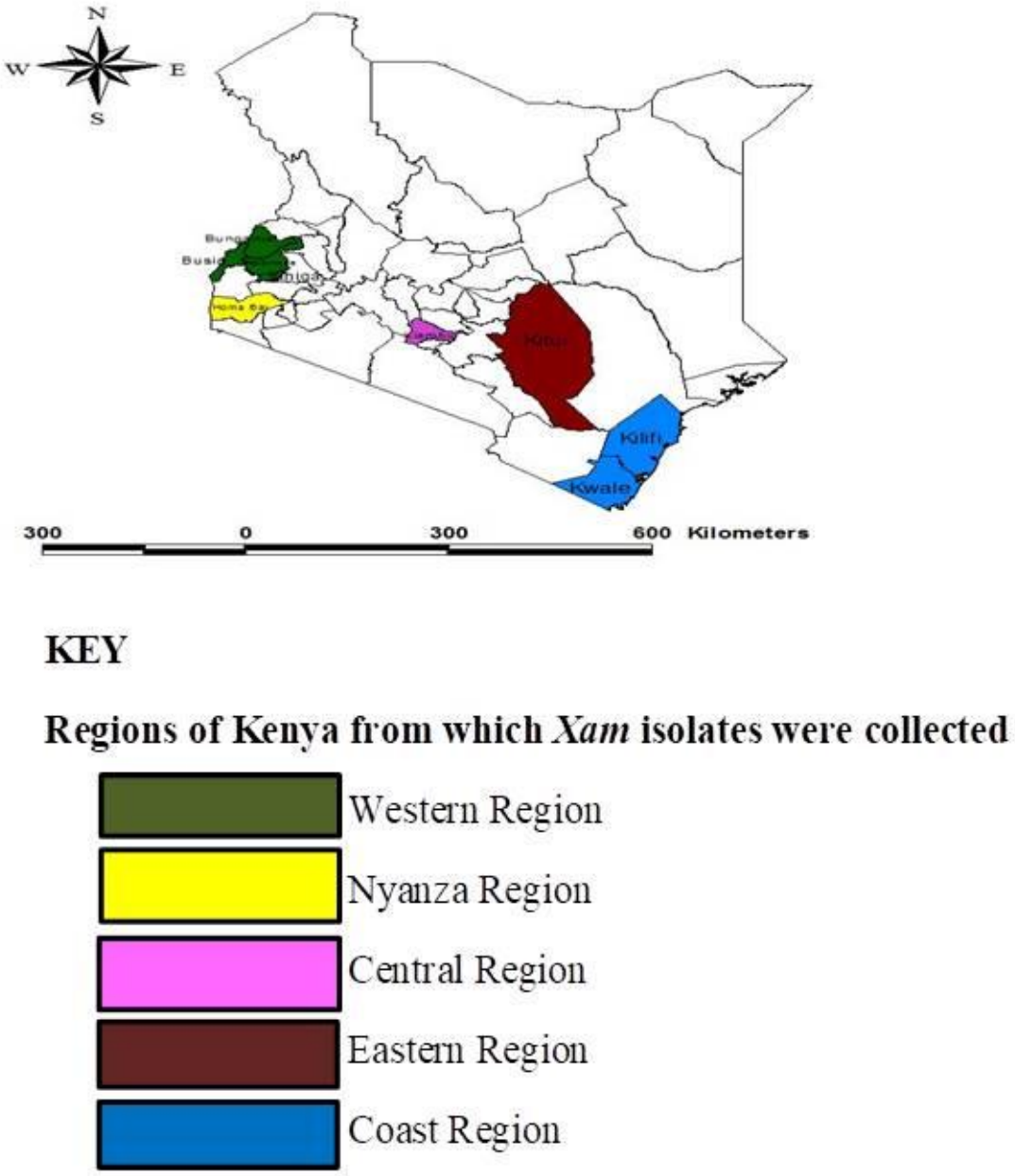

Figure 3.1: Map showing origin of Xam isolates used in this study

Table 3.1: **Summary of the regions and Counties from which cassava leaf samples showing bacterial blight symptoms were collected from for use in this study**

Region	County	Number of leaf samples collected	Latitude (°)	Longitude (°)	Altitude (m.a.s.l)
Western	Kakamega	7 (Xam1-W, Xam2-W, Xam3-W, Xam4-W, Xam5-W, Xam6-W and Xam18-W)	0.37798	34.81504	1610
	Vihiga	5 (Xam7-W, Xam8-W, Xam9-W, Xam10-W and Xam11-W)	0.38552	34.14364	1306
	Bungoma	2 (Xam12-W and Xam13-W)	0.59643	34.47245	1345
	Busia	4 (Xam14-W, Xam15-W, Xam16-W and Xam17-W)	0.59035	34.47245	1164
Eastern	Kitui	1 (Xam19-E)	1.33707	38.01816	1183
Nyanza	Homabay	4 (Xam20-N, Xam21-N, Xam22-N, Xam23-N and Xam24-N)	S00.5564	E034.41292	1311
Central	Kiambu	1 (Xam25-CE)	S01.09460	E037.01338	1466
Coast	Kilifi	6 (Xam26-C, Xam27-C, Xam28-C, Xam29-C, Xam30-C and Xam31-C)	S03.93400	E039.73534	38
	Kwale	2 (Xam32-C and Xam33-C)	S04.30731	E039.51998	90

m.a.s.l = metres above sea level (°) =degrees

3.2 Isolation of bacteria

Isolation of *Xam* was done as described by Verdier *et al.* (1998) and Ogunjubi *et al*. (2008). Necrotic portions of the CBB-infected leaves were macerated into small pieces of about 2 mm^2 and transferred into sterile 15 ml falcon tubes. About 5 ml YPG-CC (yeast extract (1%), peptone (1%), glucose (1%), agar, cephalexin (50 mg l^{-1}) and cycloheximide (150 mg l^{-1}), pH 7.0). Liquid semi-selective media was added and incubated for 48 hours at 28 °C. The bacterial suspension was diluted serially to 10^{-3}, and aliquots of 0.1 ml of each of the 10-fold dilution samples transferred onto YPGA-CC medium (yeast extract (1%), peptone (1%), glucose (1%), agar (1.5%), pH 7.0) supplemented with cephalexin (50 mg l^{-1}) and cycloheximide (150 mg l^{-1}). The Petri dishes were incubated at 28 °C for 48 hours. Single colonies were selected and purified by re-streaking on YPGA-CC, grown at 28 °C for 48 hours and kept at -20 °C for storage in 30% glycerol.

3.3 Phenotypic characterization of *Xam* isolates

Bacterial suspensions of each isolate were streaked onto plates of YPGA-CC selective media. The plates were incubated at 28 °C for 48 hours followed by morphological characterization. All the isolates were observed on YPGA-CC plates for colony characteristics including colony color, form, elevation, size, surface, edges, structures, growth rate and texture as described by Bradbury (1979) and Fahy *et al*. (1983). Appendix 1 shows the characteristics used and their scores.

3.3.1 Gram staining

Gram staining and microscopy were carried out to determine if the cultures were gram negative or positive. Staining was performed following the method described by Bradbury (1978). The smear of each isolate was made by stirring some bacterial cells obtained from a 24-hour old culture in a drop of sterile distilled water on a glass slide using sterile inoculating wire loop. The smear was air-dried; heat fixed, followed by flooding with crystal violet solution and allowed to stand for 1 minute. The slides were rinsed with water for 5 seconds, covered with Gram's iodine and allowed to stand for 1 minute and rinsed with water for 5 seconds. The stained preparation

was then rinsed with 70 percent ethanol until the color ceased to come out. . This was followed by rinsing with sterile distilled water for 5 seconds. Counterstaining was done using safranin red for 4 minutes and rinsed with sterile distilled water for 5 seconds. Blot drying was done with bibulous paper and examined under oil immersion. The reference culture used was *Escherichia coli* obtained from the stock collections of the Department of Biochemistry, University of Nairobi.

3.3.2 Potassium hydroxide (KOH) solubility test

Potassium hydroxide (KOH; 3%) solubility test was done to confirm the Gram stain reaction as described by Mortensen *et al*. (2005). A sterile toothpick was used to fetch a 24-hour bacterial isolates from a pure colony and each was mixed with 15 µl of 3% KOH aqueous solution on a clean glass slide. After mixing, a tooth pick was raised a few centimetres from a glass slide to observe a strand of viscid mucous material as described by Gregersen (1978). If the strand of viscid material was observed, the bacterial isolate in question was regarded as Gram-negative. Lack of strands of viscid material was recorded as Gram-positive.

3.4 Molecular characterization of *Xam* isolates

Out of the 26 *Xam* isolates obtained, 19 were selected based on hierarchical cluster analysis from morphological characterization for genetic diversity studies.

3.4.1 Preparation of broth culture

A single colony of each *Xam* isolate from the subculture on the YPGA-CC plate was inoculated aseptically into 10 ml suspension in 50 ml falcon tubes and incubated in a shaker incubator at 28 °C for 48 hours at speed of 220 rpm. Then, 500 µl of each of the bacterial cultures was mixed with 500 µl of glycerol solution and stored at 4 °C for future subcultures while 2 ml was used for DNA extraction.

3.4.2 Extraction of DNA from broth cultures of *Xam* isolates

Extraction of DNA was carried out following the DNeasy® mini kit protocol (Qiagen, 2013). The two milliliters broth cultures of *Xam* isolates were put into an Eppendorf tube and re-suspended, vortexed and centrifuged following the protocol of the manufacturers' recommendations (Qiagen, 2013). The eluted DNA was stored at -20 °C for further use.

3.4.3 Agarose gel electrophoresis

The quality and integrity of genomic DNA was analyzed on 1% (w/v) agarose gel electrophoresis. Agarose gel was prepared by boiling 1.0 g agarose (Sigma, St. Louis, USA) in 100 ml of 1 × TAE buffer. The boiled agarose solution was allowed to cool to about 60 ºC prior to addition of Gel Red (Biotium, South Africa) to a final concentration of 0.5µg/ ml. The solution was poured into the casting tray and a comb was inserted. After polymerization (about 30 minutes), the tray containing the gel was transferred into the electrophoresis chamber with the slots facing the cathode and 1 × TAE buffer was added to completely immerse the gel. Two microlitres of 6× DNA loading dye (Thermo scientific, USA) was mixed with 2 µl DNA of each of the samples and loaded onto the wells in the gel. The gel was separated at 80 V for 45 minutes, visualized under a UV transilluminator (Herolab, Wiesloch, Germany) and photographed.

3.4.4 Detection of *Xam* in PCR

The DNA extracted from cultures of bacterial isolates was subjected to PCR analysis using *Xam*-specific primers XV (5'-TTC-GGC-AAC-GGC-AGT-GAC-CAC-C-3') and XK (5'-TCA-ATC-GGA-GAT-TAC-CTG-AGG-G-3') in a MJ Mini™ personal Thermal Cycler (BIO-RAD, Singapore). PCR reactions were performed using Accupower®PCR premix in a total volume of 20 µl thin-walled Accupower™ PCR tube (USA Bioneer, Inc.) using 1µl of 1.5 mM $MgCl_2$, 4 µl of buffer 5X, 0.5 µl of 0.5 mM dNTPs, 0.5 µl of 10 µM of each primer, 0.5 µl of 1.25 units of *Taq* polymerase, 2 µl of isolated DNA and 11.5 µl of sterile double-distilled water. The PCR cycling conditions were an initial denaturation at 95 ºC for 2 minutes followed by 30 cycles of 94 ºC for 30 seconds (denaturation), 55 ºC of 1 minute (annealing) and 72 ºC for 1 minute

(extension) and a final extension of 5 minutes. The PCR products were electrophoresed on a 1% (w/v) agarose gel in 1× TAE buffer containing 0.5µg/ ml of Gel Red and visualized in a UV transilluminator.

3.4.5 Repetitive elements based PCR (rep-PCR)

A total of 19 *Xam* isolates were analyzed. PCR-amplification reactions were performed using primer pair: REP1R-2 (5′-IIIICGICGICATCIGGC -3′) and REP2-2 (5'–ICGICTTATCIGGCCTAC -3') as described by Louws *et al*. (1999). PCR amplifications were carried out in a 25 µl reaction volume consisting of 4 µl of 10X PCR buffer, 0.5 µl of each primer, 0.2 µl of *Taq* DNA polymerase (Qiagen), 0.5 µl of dNTPs, 1 µl of $MgCl_2$, 16.3 µl of double-distilled water and 2 µl of bacterial DNA. A non-template reaction was included as a negative control. PCR reactions were performed in a MJ Mini^{TM} personal Thermal Cycler (BIO-RAD, Singapore) using the following conditions: initial denaturation at 95 °C for 7 minutes, followed by 30 cycles of 94 °C for 1 minute (denaturation), 44 °C for 1 minute (annealing) and 65 °C for 8 minutes (extension) with a final extension at 65 °C for 15 minutes. The PCR products were electrophoresed on a 1.5 % (w/v) agarose gel in 1× TAE buffer containing 0.5µg/ ml of Gel Red and visualized in a UV transilluminator. All PCR reactions were repeated at least twice for each sample to verify the consistency of the patterns.

3.5 Data analysis

Phenotypic and cultural characteristics of Xam colonies were scored numerically and the data obtained was subjected to a hierarchical cluster analysis, using statistical package for social sciences (IBM SPSS Statistics, 2015) version 23.0. Genomic fingerprint comparisons among bacterial isolates were performed by measurement of band sizes. Only clear and reproducible bands ranging from 250 to 2000 bp were scored and recorded for each isolate. Bands generated by the rep-PCR analysis were converted into a two-dimensional binary matrix (1, presence of a band; and 0, absence of a band) using Jaccard (J) coefficient, which do not consider the negative similarities. The matrix was analyzed by Popgene package. Unweighted Pair Group Method with

Arithmetic mean (UPGMA) algorithm was used to perform hierarchical cluster analysis and construct a dendrogram.

CHAPTER FOUR

RESULTS

4.1 Isolation and biochemical characteristics of *Xam* isolates

A total of 26 bacterial isolates were obtained from 33 cassava infected samples collected from farmer's fields (Table 2). All the 26 bacterial isolates produced mucoidal colonies on YPGA-CC selective media (Figure 4A). Biochemical tests of the bacterial isolates indicated that all the isolates were Gram-negative (Figure 4B and produced Gram-negative mucoidal thread-like rods in 3% KOH (Figure 4C).

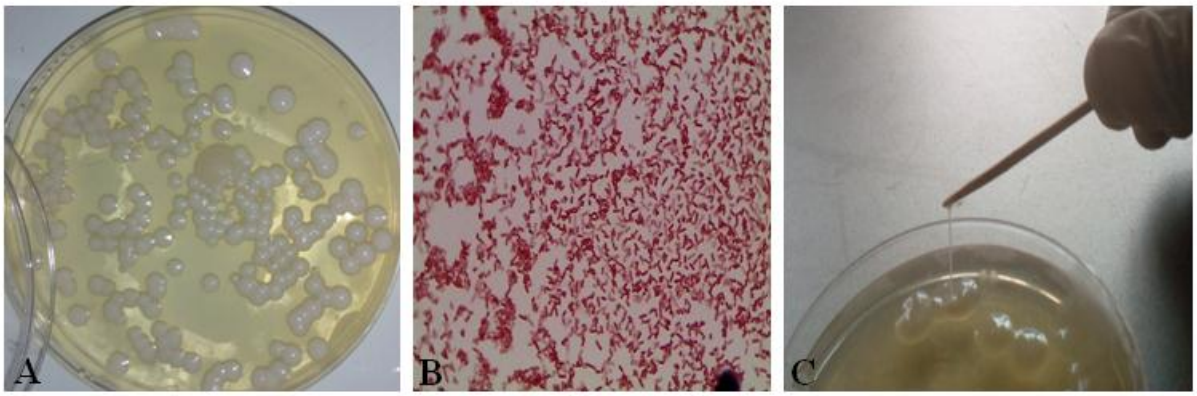

Figure 4.1: Colonies of isolated Xam from infected leaf samples and results of biochemical test

(A) colony of Xam on YPGA-CC; (B) microscopic view of Gram stained Xam isolate; and (C) mucous thread produced by Gram-negative bacteria on 3% KOH.

4.2 Phenotypic characterization of *Xam* isolates

4.2.1 Phenotypic characteristics of *Xam* isolates

Colonies of cultured *Xam* isolates appeared after 48 hours on YPGA-CC selective media.Differences between isolates were verified using phenotypic characteristics and the scores

recorded for each isolate (Table 4.1) The colonies of the isolates were white/yellow,mucoid and circular/round on YPGA-CC (Figure 4.2). Colony color varied from white to cream in all the isolates except one isolate (Xam12-W) that was yellow (Figure 4.2).

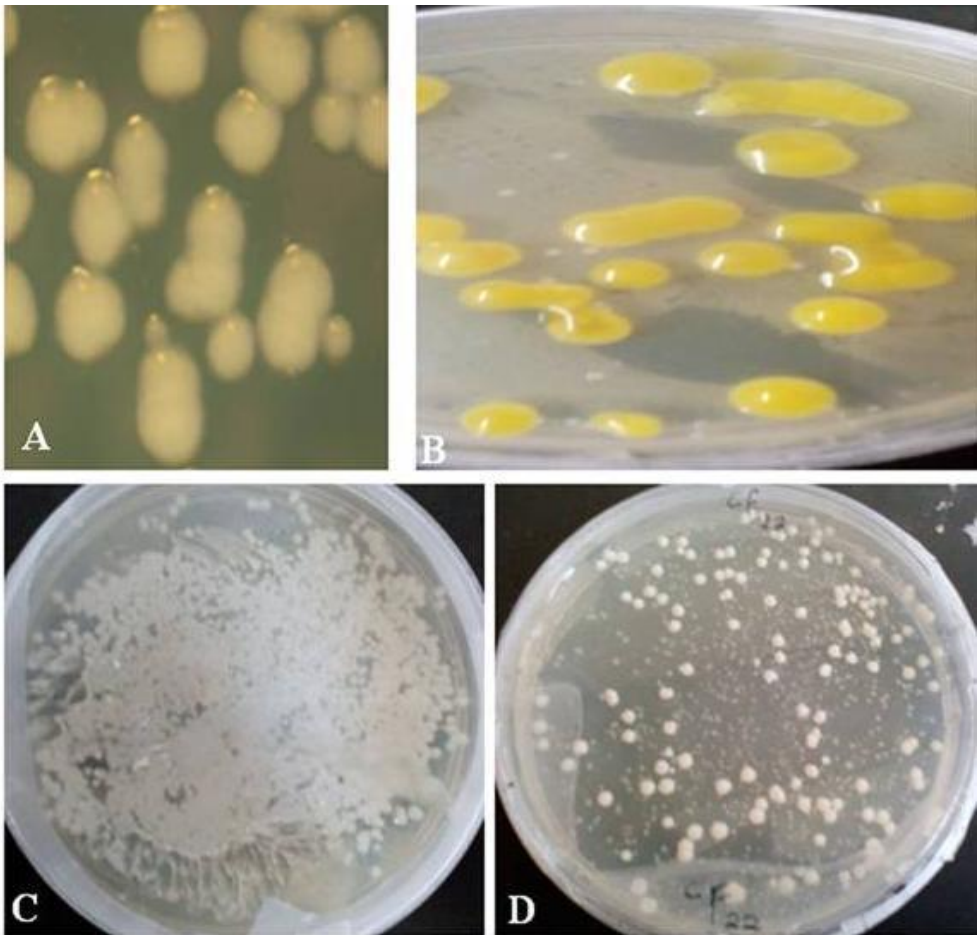

Figure 4.2: Colonies of Xam after 48 hours of culture on YPGA-CC selective media

(A) Xam1-W: white with creamy and entire margins, circular, large, smooth, mucoid; (B) Xam12-W: pale yellow with smooth and entire margins, circular, large, raised; (C) Xam21-N; white with smooth and entire edges, Umbonate, medium, opaque, mucoid and (D) Xam25-C: white with smooth and entire margins, circular, umbonate,mucoid.

Table 4.1: Morphological/cultural characteristics of Xam isolates on Yeast Peptone Glucose Agar Medium

No.	Isolate	Cultural/morphological characteristics
1	Xam1-W	Circular shape, white, umbonate, large size, smooth surface, entire edge, translucent, moderate growth, mucoid texture
2	Xam2-W	Irregular shape, white, flat, small size, rough surface, undulate edge, translucent, less growth, moist texture
3	Xam3-W	Circular shape, white, flat, large size, rough surface, undulate edge, opaque, high growth, moist texture
4	Xam4-W	Circular shape, white, flat, small size, rough surface, undulate edge, translucent, less growth, moist texture
5	Xam5-W	Circular shape, white, umbonate, medium size, smooth surface, entire edge, translucent, moderate growth, mucoid texture
6	Xam6-W	Circular shape, white, umbonate, large size, smooth surface, entire edge, translucent, very high growth, mucoid texture
7	Xam7-W	Circular shape, white, umbonate, large size, smooth surface, entire edge, translucent, very high growth, mucoid texture
8	Xam8-W	Circular shape, white, flat, small size, smooth surface, entire edge, translucent, very high growth, moist texture
9	Xam9-W	Circular shape, white, flat, small size, smooth surface, entire edge, translucent, high growth, moist texture

10	Xam10-W	Circular shape, white, flat, small size, smooth surface, entire edge, translucent, high growth, moist texture
11	Xam11-W	Circular shape, white, flat, medium size, smooth surface, entire edge, translucent, very high growth, moist texture
12	Xam19-E	Circular shape, white, flat, small size, rough surface, entire edge, translucent, moderate growth, mucoid texture
13	Xam20-N	Circular shape, white, umbonate, medium size, smooth surface, entire edge, translucent, high growth, mucoid texture
14	Xam25-C	Circular shape, white, umbonate, small size, smooth surface, entire edge, translucent, very high growth, mucoid texture
15	Xam22-N	Circular shape, white, umbonate, large size, smooth surface, entire edge, opaque, less growth, mucoid texture
16	Xam24-N	Circular shape, white, umbonate, small size, smooth surface, entire edge, opaque, very high growth, moist texture
17	Xam23-N	Circular shape, white, umbonate, large size, smooth surface, entire edge, opaque, very high growth, mucoid texture
18	Xam21-N	Circular shape, white, umbonate, medium size, smooth surface, entire edge, opaque, very high growth, mucoid texture
19	Xam26-C	Circular shape, white, flat, small size, smooth surface, entire edge, translucent, high growth, moist texture
20	Xam27-C	Circular shape, white, flat, small size, smooth surface, entire edge,

		transparent, very high growth, moist texture
21	Xam28-C	Circular shape, white, flat, small size, smooth surface, entire edge, translucent, high growth, moist texture
22	Xam29-C	Circular shape, white, umbonate, medium size, smooth surface, entire edge, translucent, high growth, moist texture
23	Xam30-C	Circular shape, white, umbonate, medium size, smooth surface, entire edge, translucent, high growth, moist texture
24	Xam31-C	Circular shape, white, umbonate, small size, smooth surface, entire edge, translucent, moderate growth, moist texture
25	Xam32-C	Circular shape, white, umbonate, large size, smooth surface, entire edge, translucent, very high growth, mucoid texture
26	Xam33-C	Circular shape, white, umbonate, large size, smooth surface, entire edge, translucent, very high growth, mucoid texture

4.2.2 Clustering of *Xam* isolates based on phenotypic characteristics

Phylogenetic analysis of the 26 *Xam* isolates resulted in two major clusters I and II, at a similarity level of 75% (Figure 4.3). Cluster I comprised fifteen isolates (Xam22-N, Xam31-C, Xam1-W, Xam8-W, Xam5-W, Xam30-C, Xam29-C, Xam21-N, Xam24-N, Xam25-C, Xam23-N, Xam7-W, Xam33-C and Xam32-C) (Figure 4.1; Table 4.2) obtained from Coast, Nyanza and western regions. Isolates in this sub-cluster were characterized by small, white, circular, smooth, moist and translucent colonies. Cluster II comprised ten isolates (Xam4-W, Xam19-E, Xam26-C, Xam28-C, Xam9-W, Xam10-W, Xam8-W, Xam11-W, Xam3-W and Xam27-C) (Figure 4.3)

obtained from Coast, eastern and western regions of Kenya. Isolates in this sub-cluster were characterized by small, white, circular, smooth, mucoid translucent and elevated colonies. One isolate, Xam 2-W obtained from western Kenya was not clustered by the phenotypic analysis. It was characterized by small, white, circular, dry, flat and translucent colonies.

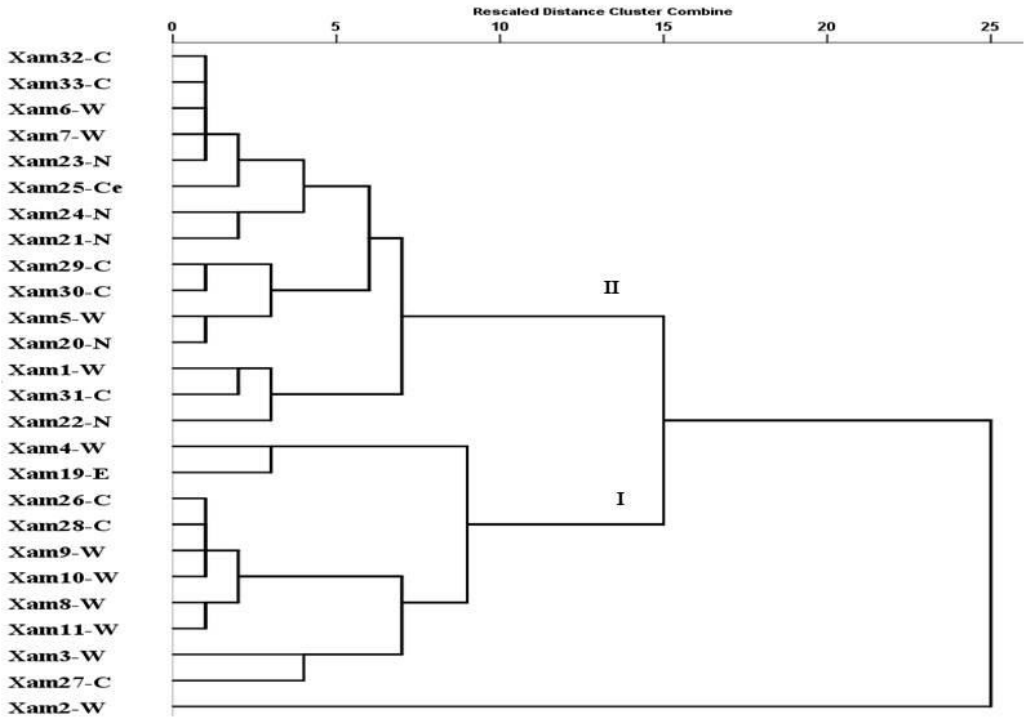

Figure 4.3: Dendrogram of phenotypic diversity of 26 *Xam* isolates generated using phenotypic/cultural characteristics

Table 4.2: Phenotypic characters separating Xam isolates into clusters and sub-clusters

Cluster	Similarity coefficient (%)	Isolates	Distinguishing colony characteristics
I	93 – 98	15 (Xam22-N, Xam31-C, Xam1-W, Xam8-W, Xam5-W, Xam30-C, Xam29-C, Xam21-N, Xam24-N, Xam25-C, Xam23-N, Xam7-W, Xam33-C and Xam32-C)	Small, white, circular, smooth, moist and translucent colonies
II	96 – 98	10 (Xam4-W, Xam19-E, Xam26-C, Xam28-C, Xam9-W, Xam10-W, Xam8-W, Xam11-W, Xam3-W and Xam27-C)	Small, white, circular, smooth, mucoid translucent and elevated colonies

4.2.3 Principal component analysis (PCA)

Principal component analysis of *Xam* isolates based on colony morphological characteristics, revealed four principal components (PC) on a two dimensional plot (Figure 4.4), which had eigen values of between 0.816 and 3.086 and accounted for 80.8% of the total variation (Table 4.3). The first PC accounted for 38.6% whereas the second, third, and forth PC axes accounted for 19.6%, 12.4% and 10.2%, respectively. The first PC was positively associated with characters such as form, color, size and elevation; the second PC was associated with color and elevation; the third was associated with form and color while the forth was associated with color-related characters.

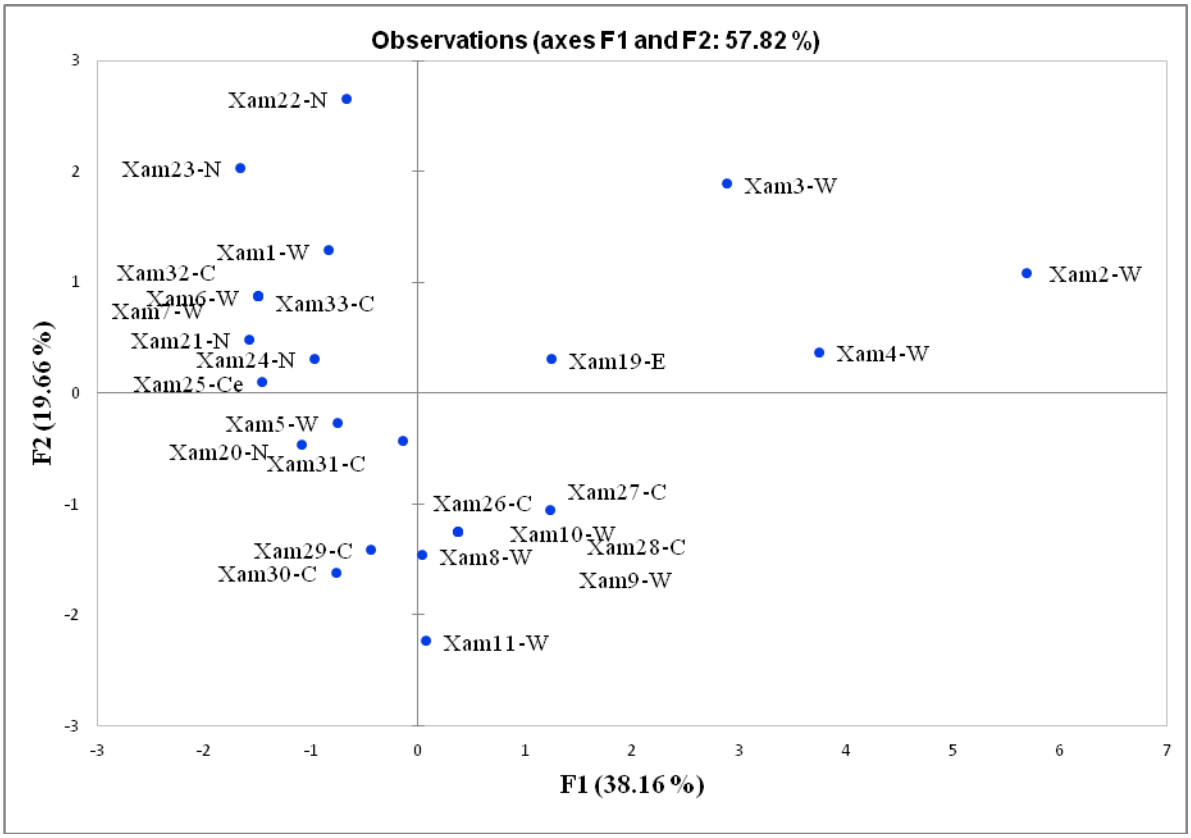

Figure 4.4: Principal component analysis of *Xam* isolates from Kenya based on phenotypic/cultural characteristics using XLSTAT. F – Factor scores

Table 4.3: Eigen values, total variation and cumulative variation of the four principal components for the 26 Xam isolates

Principal component	Eigen value	Variation %	Cumulative %
1	3.086	38.577	38.577
2	1.572	19.647	58.224
3	0.993	12.409	70.633
4	0.816	10.206	80.839

4.3 Genetic diversity of *Xam* isolates assessed by rep-PCR genomic fingerprinting

4.3.1 Rep-PCR amplification of *Xam* isolates

The genetic diversity within Kenyan *Xam* population was assessed using rep-PCR method. All isolates produced characteristic fingerprint pattern with bands ranging between 300 and 3000 bp and their numbers ranged from 1 to 6 bands per isolate (Figure 4.5). The same fingerprints were observed when the PCR was repeated at least three times. Minor light amplification bands which were not reproducible were not taken into account in data analysis. Majority of isolates revealed complex banding pattern, with only four isolates exihibiting less than four bands. Isolates Xam19-E and Xam24-N generated a low complexity pattern with one and three bands, respectively.

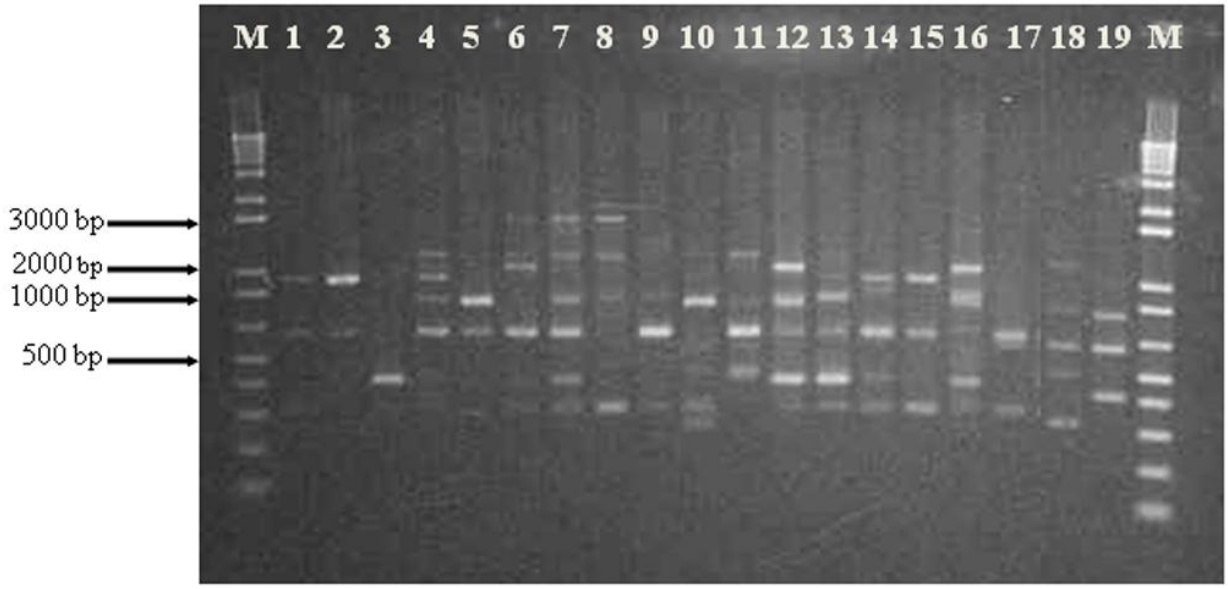

Figure 4.5: Agarose gel electrophoresis of rep-PCR fingerprinting patterns from genomic DNA of Xam isolates obtained from infected cassava

Lanes M represent 1 kb molecular weight marker (Fermentas); Lanes 1 (Xam1-W), 2 (Xam24-N), 3 (Xam19-E), 4 (Xam26-C), 5 (Xam8-W), 6 (Xam6-W), 7 (Xam2-W),8 (Xam22-N), 9 (Xam27-C), 10 (Xam30-C), 11 (Xam20-N), 12 (Xam16-W), 13 (Xam16-W), 14 (Xam5-W), 15 (Xam21-N), 16 (Xam23-N), 17 (Xam4-W), 18 (Xam11-W) and 19 (Xam25-Ce) represent Xam isolates from Western (W), Nyanza (N), Coast (C) , Eastern (E) and Central (Ce) regions of Kenya.

4.3.2 Clustering of *Xam* populations

To examine the relatedness of Kenyan *Xam* populations, rep-PCR fingerprints for selected 19 isolates were used to construct a dendrogram based on the similarity in the polymorphisms of the DNA fragments generated. Overall, limited genetic variability was observed among most of the assessed *Xam* populations in Kenya, with the exception of isolate Xam-19E that appeared to differ from the rest of the isolates (Figure 4.5). The dendrogram constructed from the UPGMA of similarities between rep-PCR profiles of Xam (Figure 4.6) had two main cluster. Cluster-A and cluster-B. Cluster-A was divided into two sub-clusters. The sub-cluster-I was generated by 17

isolates that formed group 1 and group 2. Group 1 consisted of Xam1-W, Xam24-N, Xam6-W, Xam21-W, Xam26-C, and Xam11-W. Group 2 consisted of Xam8-W, Xam27-C, Xam10-W, Xam16-W, Xam15-W, Xam2-W, Xam23-W, Xam25-Ce, Xam5-W, Xam4-W , and Xam25-N all that belonged to the Western, Coast and Central regions of Kenya. The sub-cluster-II was formed by Xam30-C and Xam22-N isolates which belonged to the Coast and Nyanza regions. Cluster-B consisted of only one isolate, Xam19-E that belonged to the Eastern region.

At 10% similarity coefficient level, isolate Xam-19E that had unique gel fingerprints (only single band) did not group with the rest of the isolates that randomly clustered in two clusters. The UPGMA analysis generated random clusters regardless of the geographical origin of the isolates (Figure 4.6). For example, isolate Xam-11W from Vihiga County in Western region clustered together with isolate Xam-26C from Kilifi County in coastal Kenya. Isolate Xam-5W (from Kakamega County in western Kenya) clustered with isolate Xam-25Ce (from Kiambu County in central Kenya) (Figure 4.6). High genetic similarity coefficients were recorded for *Xam* isolates from Western and Coastal regions (Table 4.3).

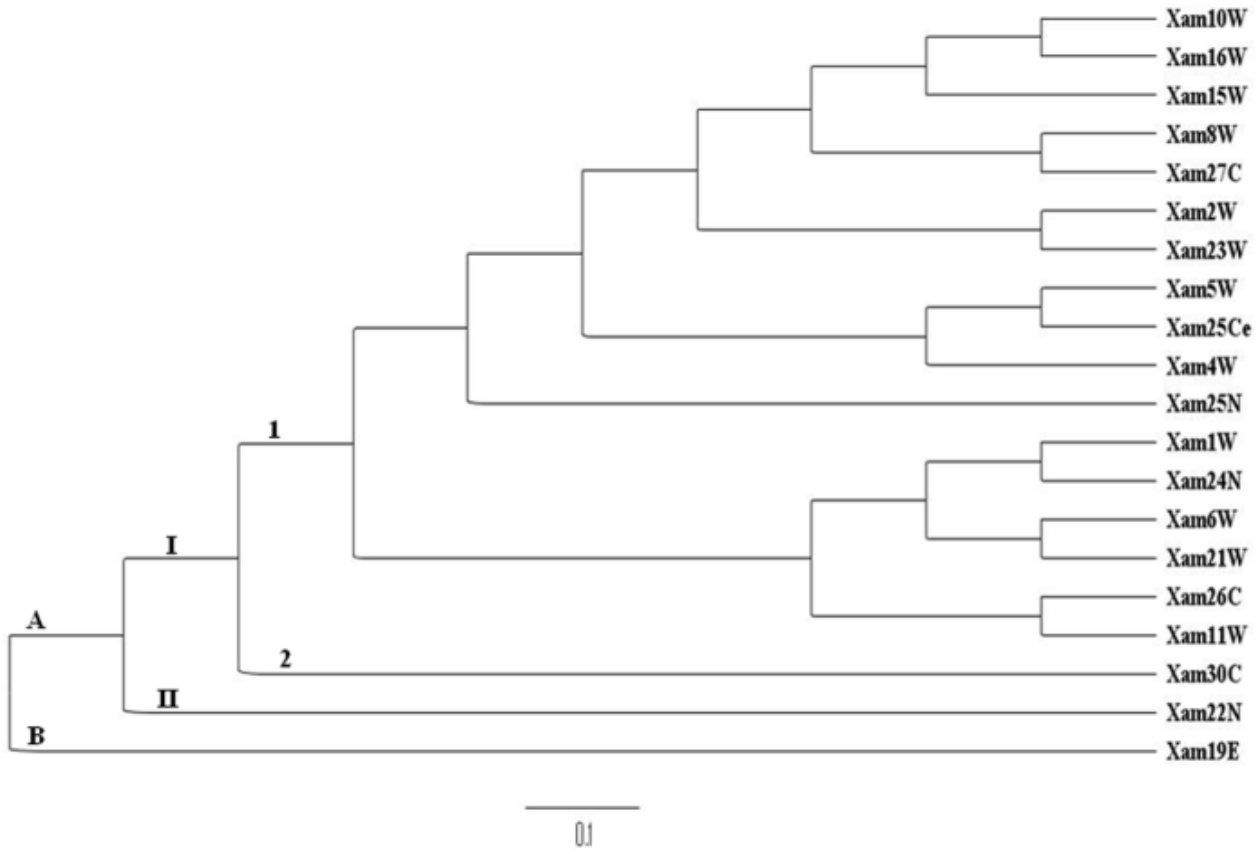

Figure 4.6: Unweighted pair group method, arithmetic average (UPGMA) dendrogram constructed from rep-PCR fingerprinting data of *Xam* isolates from different regions in Kenya

Table 4.4: Similarity of Xam isolates based on combined analysis of Jaccard matrix

	Xam1W	Xam24N	Xam19E	Xam26C	Xam8W	Xam6W	Xam2W	Xam22N	Xam27C	Xam30C	Xam25N	Xam10W	Xam16W	Xam15W	Xam5W	Xam21W	Xam23W	Xam4W	Xam11W	Xam25Ce
Xam1W	1	1	0	0.5	0.25	0.5	0.143	0	0.25	0	0.25	0.2	0.2	0.2	0.5	0.667	0.167	0.333	0.4	0.25
Xam24N		1	0	0.5	0.25	0.5	0.143	0	0.25	0	0.25	0.2	0.2	0.2	0.5	0.667	0.167	0.333	0.4	0.25
Xam19E			1	0	0	0	0.167	0	0	0	0.333	0.25	0.25	0.25	0.25	0	0.2	0	0.2	0.333
Xam26C				1	0.4	0.333	0.429	0.167	0.4	0.167	0.4	0.333	0.333	0.333	0.333	0.4	0.5	0.2	0.5	0.167
Xam8W					1	0.4	0.5	0.2	1	0.5	0.2	0.75	0.75	0.75	0.4	0.5	0.6	0.667	0.333	0.5
Xam6W						1	0.429	0.4	0.4	0.167	0.167	0.333	0.333	0.333	0.6	0.75	0.286	0.5	0.286	0.4
Xam2W							1	0.5	0.5	0.286	0.5	0.667	0.667	0.667	0.429	0.286	0.833	0.333	0.375	0.5
Xam22N								1	0.2	0.2	0.2	0.167	0.167	0.167	0.167	0.2	0.333	0.25	0	0.2
Xam27C									1	0.5	0.2	0.75	0.75	0.75	0.4	0.5	0.6	0.667	0.333	0.5
Xam30C										1	0	0.4	0.4	0.4	0.167	0.2	0.333	0.25	0.333	0.2
Xam25N											1	0.4	0.4	0.4	0.4	0.2	0.6	0.25	0.333	0.5
Xam10W												1	1	1	0.6	0.4	0.8	0.5	0.5	0.75
Xam16W													1	1	0.6	0.4	0.8	0.5	0.5	0.75
Xam15W														1	0.6	0.4	0.8	0.5	0.5	0.75
Xam5W															1	0.75	0.5	0.5	0.5	0.75
Xam21W																1	0.333	0.667	0.333	0.5
Xam23W																	1	0.4	0.429	0.6
Xam4W																		1	0.167	0.667
Xam11W																			1	0.333
Xam25Ce																				1

4.3.3 Principal component analysis (PCA)

Genetic relationship of individual *Xam* isolates was analyzed using PCA (Figure 4.7). The PC1 and PC2 acccounted for 38.58% and 19.65% of the total variance, respectively. Groupings of the 19 *Xam* isolates was not based on geographical region of collection (Figure 4.7). The PCA showed close clustering of the isolates except three isolates (Xam19-E from Eastern and two isolates Xam4-W and Xam23-W from Western region) which were clustered away from the rest of the isolates.

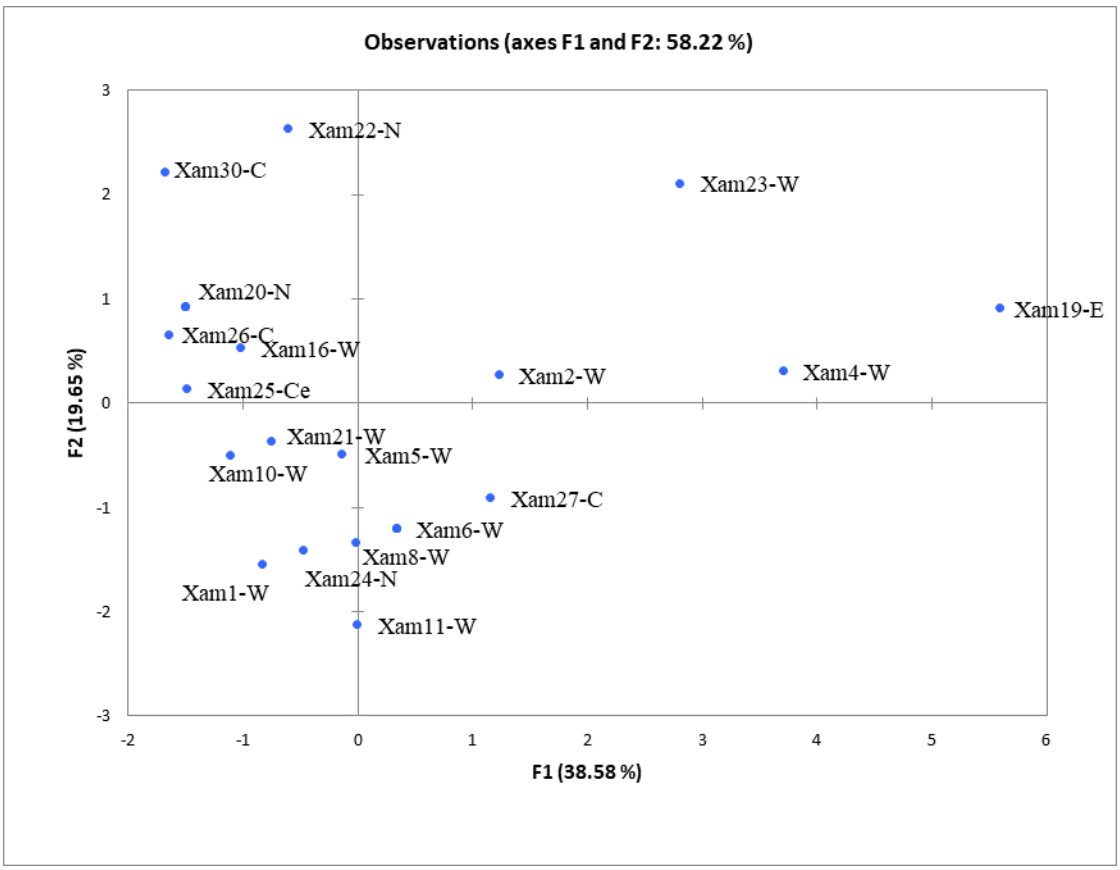

Figure 4.7: Biplot representation of observations in the PCA space on the axes PC1 and PC2 showing the correlation of variables and observations of the *Xam* isolates

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECCOMMENDATIONS

5.0 Discussion

The isolation of *Xam* from the infected leaves of cassava and culturing them in the selective media was successful because colony formation was evident in semi-selective media. Colonies of cultured *Xam* isolates appeared after 48 hours on YPGA-CC selective media. The results of this study reveal the presence of cassava bacterial blight caused by *Xanthomonas axonopodis* pv. *manihotis* in major cassava growing regions in Kenya. The symptoms observed in the leaves and stems were similar to those described in other African and Latin American countries (Daniel *et al*., 1985).

Phenotypic studies were carried out to find out the variation of the 26 *Xam* isolates. Colony color varied from white to cream in all the isolates except one isolate (Xam12-W) that was yellow. The results are in agreement with the report by Verdier *et al*. (2004) who observed that *Xam* colonies appear creamy and white in color and form distinct colonies. In addition, Trujillo *et al*. (2014) reported that *Xam* cultured on selective media is distinguished by white and cream colonies. Reports of variations in color, shape, elevation and contour (margin) among *Xam* strains have also been documented in previous studies (Ogunjobi *et al*., 2010, Nguyen and Kwoh, 2015; Adhikari *et al*., 1999). Phylogenetic analysis based on morphological characteristics revealed random clustering of the Kenyan *Xam* isolates from the different geographical regions. Differences in morphological characteristics in *Xanthomonas campestris* and *Xanthomonas ampelina* have also been reported by McGuire and Jones (1987) and Willems *et al*. (1987), respectively.

Pathogen diversity can be done by combining both genotypic differences between isolates and phenotypic analyses. PCR-based approaches have been used to study genetic diversity of phytopathogenic bacteria and generate evidence of their ecological distribution and evolution (Adhikari *et al*., 1999; Kumar *et al*., 2004). Repetitive DNA primers corresponding to conserved motifs in bacterial repetitive elements and PCR are used to show that REP DNA sequences are

widely distributed in phytopathogenic Xanthomonas (Lee et al., 2008). In this study, the genetic diversity within Kenyan *Xam* population was assessed using rep-PCR technique. All isolates produced characteristic fingerprint pattern ranging from 1 to 6 bands per isolate and the similarity of the fingerprints varied from 10 to 90%. The size and distribution of bands were distinct and unique to each isolate. The same fingerprints were observed when the PCR was repeated at least three times, demonstrating the reproducibility of this technique for genetic studies of *Xam*. Some minor light amplification bands were also not reproducible, suggesting non-specific binding and such bands were not taken into account. This is in agreement with the study done by Janssen *et al*. (1996) and Restrepo *et al*. (2000), who confirmed the usefulness of rep-PCR in characterizing bacterial populations and its applicability to the study of *Xam* populations.

Majority of the isolates revealed multiple banding pattern, with only four isolates exihibiting less than four bands. The results from the present study confirm reports by Restrepo *et al*. (1997) who demonstrated high discriminatory power of rep-PCR when characterizing *Xam* populations with low levels of genetic diversity in Colombia. A dendrogram constructed based on the similarity in the polymorphisms of the DNA fragments examined the relatedness of Kenyan *Xam* populations. Overall, limited genetic variability was observed among most of the assessed *Xam* populations in Kenya, with the exception of isolate Xam-19E that appeared to differ from the rest of the isolates. At 10% similarity coefficient level, isolate Xam-19E that had unique gel fingerprints (only single band) grouped solely while the rest of isolates randomly clustered into two clusters. The results from this study confirm the existence of limited genetic diversity within the current populations of *Xam* in Kenya. The lack of a correlation between rep-PCR groupings and geographic origin of isolates is an indication that this bacterium has spread among different regions in Kenya. The results suggest that these isolates have not as yet diversified at the genotypic level. The genetic diversity could not differentiate the yellow strain from the white and cream strains that cause cassava bacterial blight disease.

The random clustering of the *Xam* isolates from different geographical regions could be due to the continued use of a diseased cassava stem during propagation. It is possible that a single isolate

was introduced in Kenya and simultaneously spread to other parts. This is in agreement with the study done by Onyeka et al. (2008), who confirmed that the high incidence of CBB in Nigeria was due to the cultivation of single and often susceptible but popular local cultivar by the farmers. Moreover, the farmer practice of obtaining plant materials from near and far, without taking precautions, such as certification of the planting materials to avoid carrying infected stem cuttings and moving that to another place over a long distance could have perpetuated the same isolate. However, the exact source of cassava bacterial blight in Kenya still remains unknown. These results contradict studies from other parts of the world such as Colombia by Restrepo *et al*. (1997) who reported that *Xam* causing bacterial blight exhibit a high level of genotypic variability and geographical differentiation. Collectively, the results presented gave an insight into the Kenyan *X. axonopodis pv. manihotis* existence.

5.1 Conclusions

From the findings of this study it can be concluded that:

1. There is presence of *Xam* pathogen in different cassava growing regions.
2. There exist cassava bacterial blight in all cassava growing regions of Kenya, which should be considered as significant threat to food security particularly among the subsistence and smallholder cassava farmers.
3. The rep-PCR results from this study have demonstrated limited genetic diversity within *Xam* isolates that were isolated from cassava in Kenya.

5.2 Recommendations

Based on the findings of this study, the following recommendations were made:

1. Due to the limited genetic diversity of the *Xam* isolates, there is need for development and wide use of resistant cultivars that will effectively control the disease.
2. There is need to re- evaluate other primers available for DNA-fingerprinting on how they reveal the interrelatedness of *Xam* isolates in Kenya.

3. Sequencing of the obtained *Xam* isolates should be done to allow the differentiation of the isolate observed with yellow colonies from those with creamy/white colonies.

REFERENCES

Abd-El-Haleem, D., Layton, A. C., & Sayler, G. S. (2002). Long PCR-ampli¢ed rDNA for PCR RFLP and rep-PCR-based approaches to recognize closely related microbial species. *Journal of Microbiological Methods, 49,*315-319.

Adhikari, T. B., Mew, T. W., & Leach, J. E. (1999). Genotypic and pathotypic diversity in *Xanthomonas oryzae pv. oryzae* in Nepal. *Phytopathology, 89(8),* 687-694.

Allem, A. C. (2002). The origin and taxonomy of cassava. *Cassava: biology, production and utilization, 1,* 1-16.

Balagopalan, C. (2002). Cassava utilization in food, feed and industry. *Cassava: Biology, production and utilization,* 301-318.

Banito, M. S. A. (2003). *Integrated control of cassava bacterial blight in West Africa in relation to eco-zones, host plant resistance and cultural practices*. Ph.D. Thesis. Institute of Plant Diseases and Protection, University of Hannover, Germany.

Boher, B., & Verdier, V. (1994). Cassava bacterial blight in Africa: the state of knowledge and implications for designing control strategies. *African Crop Science Journal, 2(4),* 505-509.

Boher, B., Kpemoua, K., Nicole, M., Luisetti, J., and Geiger, J. P. (1995). Ultrastructure of interactions between cassava and *Xanthomonas campestris pv. manihotis*: Cytochemistry of cellulose and pectin degradation in a susceptible cultivar. *Phytopathology, 85(7),* 777-788.

Bradbury, J. F. (1978). *Identification and characteristics of Xanthomonas manihotis*. In: *Terry, ER; Persley, GJ; Cook, SCA (edxs.), Workshop on Cassava Bacterial Blight in Africa, Past, Present and Future Ibadan, Nigeria. Pg. 1-4.*

Bradbury, J. (1984). *Xanthomonas*. *Bergey's manual of systematic bacteriology, Vol. 1*.

Bull, S. E., Briddon, R. W., Sserubombwe,, W. S., Ngugi K., Markham, P. G., & Stanley, J. (2006). Genetic diversity and phylogeography of cassava mosaic viruses in Kenya. *Journal of General Virology, 87(10)*, 3053-3065.

Bull, S., Karakacha, H., Briddon, R., Nzioki, S., Maruthi, M., Stanley, J., & Winter S. (2003). Occurrence of East African cassava mosaic Zanzibar virus (EACMZV) in coastal Kenya. *Plant Pathology, 52(6)*, 791.

Castiblanco, L. F., Gil, J., Rojas, A., Osorio, D., Gutiérrez, S., Muñoz- Bodnar, A., & Restrepo, S. (2013). TALE1 from *Xanthomonas axonopodis pv. manihotis* acts as a transcriptional activator in plant cells and is important for pathogenicity in cassava plants. *Molecular Plant Pathology, 14(1)*, 84-95.

Chaudhry, Z., & Rashid, H. (2011). Isolation and characterization of Ralstonia solanacearum from infected tomato plants of soan skesar valley of Punjab. *Pakistan Journal of Botany, 43(6)*, 2979-2985.

Chavez, A. L., Sanchez, T., Jaramillo,, G., Bediya J. M. I., Echeverry, J., Bolanos, E. A., Ceballos, H., & Iglesias, C. A. (2005). Variation of quality traits in cassava roots evaluated in landraces and improved clones. *Euphytica, 143(7)*, 125-33.

Cruz, C. V., Ardales E. Y., Skinner, D. Z., Talag, J., Nelson, R. J., Louws, F. J., & Leach, J. E. (1996). Measurement of haplotypic variation in *Xanthomonas oryzae pv. oryzae* within a single field by rep-PCR and RFLP analyses. *Phytopathology, 90*, 683-690.

Cubero, J., & Graham, J. H. (2002). Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Applied and Environmental Microbiology, 68(3)*, 1257 -1264.

Daniel, J. F., & Boher, B. (1985). Epiphytic phase of *Xanthomonas campestris* pathovar *manihotis* on aerial parts of cassava. *Agronomie, 5(2),* 111-116.

Dixon, A. G., Ngeve J. M., & Nukenine, E. N. (2002). Genotype× environment Effects on Severity of Cassava Bacterial Blight Disease caused by *Xanthomonas axonopodis pv. manihotis*. *European Journal of Plant Pathology, 108(8),* 763-770.

Fahy, P. C., & Persley, G. J. (1983). *Plant bacterial diseases. A diagnostic guide*. Australia: Academic Press.

Fermont, V. A., Van Asten, P., Tittonel, P., Van Wijk, M., &Giller, K. (2009). Closing the cassava yield gap: an analysis from smallholder farms in East Africa. *Field Crops Research, 112(1),* 24-36.

Food and Agricultural Organisation of the United Nations (FAO), (2013). Production of Cassava in Africa and in the World. Retrieved from: http://faostat3.fao.org/browse/Q/QC/E.

Gonzalez, C., Restrepo, S., Tohme, J., & Verdier, V. (2002). Characterization of pathogenic and nonpathogenic strains of *Xanthomonas axonopodis* pv. *manihotis* by PCR-based DNA fingerprinting techniques. *FEMS Microbiology Letters, 215,* 23–31.

Gregersen, T. (1978). Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology, 5(2),* 123-127.

Hahn, S. K., & Williams, R. J. (1973). Investigations on cassava in the Republic of Zaire. *Rapport au Commissaire d'Etat a l'Agriculture*.

Hershey, H. (2005). Cassava genetic improvement: Theory and Practice. CIAT/ Rockefeller.

Henry, G., & Westby, A. (2000). Global Cassava starch markets: Current situation and outlook. In: HowelerR.H., OatesC.G. and O'Brien G.M. (Eds), Proceedings of the International

Symposium on cassava,starch and starch Derivatives, 7-12 November,Nanning,Guangxi, China. pg.79-100

Hillocks, R. J., & Wydra, K. (2002). Bacterial, fungal and nematode diseases. *Cassava: biology, production and utilization*, 261-280.

Hulton, C. S.J., Higgins, C. F., & Sharp, P. M. (1991). ERIC sequences: a novel family of repetitive elements in the genomes of Escherichia coli, Salmonella typhimurium and other enterobacteria. *Molecular Microbiology, 5(4)*, 825-834.

International Institute of Tropical Agriculture (IITA). (2014). Cassava production and utilization in Africa. Retrieved from: http://www.iita.org/cassava.

Janssen, P., Coopman, R., Huys, G., Swings, J., Bleeker, M., Vos, P., & Kersters, K. (1996). Evaluation of the DNA fingerprinting method AFLP as a new tool in bacterial taxonomy. *Microbiology, 142(7)*, 1881-1893.

Jorge, V., Fregene, M. A., Velez, C. M., Duque, M. C., Tohme, J., & Verdier, V. (2001).QTL analysis of field resistance to *Xanthomonas axonopodis pv. manihotis* in *cassava*. *Theoretical and Applied Genetics, 101(5-6)*, 865-872.

Kawano, K. (1980). Cassava. *Hybridization of Crop Plants*, 225-233.

Kawano, K. (2003). Thirty years of cassava breeding for productivity—biological and social factors for success. *Crop Science, 43(4)*, 1325-1335.

Kumar, A., Sarma, Y. R., & Anandaraj, M. (2004). Evaluation of genetic diversity of *Ralstonia solanacearum* causing bacterial wilt of ginger using REP–PCR and PCR–RFLP. *Current Science, 87*, 1555-1561.

Lancaster, P. A., & Brooks, J. E. (1983). Cassava leaves as human food. *Economic Botany, 37(3)*, 331-348.

Lee, Y. H., Lee, S., Lee, D. H., Ji, S. H., Chang, H. Y., Heu, S., & Park, E. W. (2008). Differentiation of citrus bacterial canker strains in Korea by host range, rep-PCR fingerprinting and 16S rDNA analysis. *European Journal of Plant Pathology, 121(1)*, 97-102.

Liu, J., Zheng, Q., Ma, Q., Gadidasu, K. K., & Zhang, P. (2011). Cassava genetic transformation and its application in breeding. *Journal of Integrated Plant Biology. 53*, 552–569.

López, C. E., & Bernal, A. J. (2012). Cassava bacterial blight: using genomics for the elucidation and management of an old problem. *Tropical Plant Biology*, 5(1), 117-126.

Louws, F. J., Fulbright, D. W., Stephens, C. T., & De Bruijn, F. J. (1995). Differentiation of Genomic Structure by rep-PCR Finger-printing to Rapidly Classify Xanthomonas campestris pv. vesicatoria. *Phytopathology, 85(5)*, 528-536.

Louws, F. J., Rademaker, J. L. W., & De Bruijn, F. J. (1999). The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection, and disease diagnosis. *Annual Review of Phytopathology, 37(1)*, 81-125.

Louws, F. J., Fulbright, D. W., Stephens, C. T., & De Bruijn, F. J. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. *Applied and Environmental Microbiology, 60(7)*, 2286-2295.

Lozano, C. (1975). Bacterial blight of cassava. *Phytopathology, 21(4)*, 38-43.

Lozano, J. C. (1986). Cassava bacterial blight: a manageable disease. *Plant Disease, 70(12)*, 1989-1993.

Lozano, J. C. (1989). Outbreaks of cassava diseases and losses induced. *Fitopatologia Brasileira, 14(1)*, 7-11.

Lozano, J. C., & Laberry, R. (1982). Screening for resistance to cassava bacterial blight. *Plant Disease, 66(4)*, 316-318.

Lozano, C., & Sequeira, L. (1974). Bacterial blight of cassava in Colombia: epidemiology and control. *Phytopathology, 64(1)*, 83-88.

Lozano, J. C., & Terry, E. R. (1977). Cassava diseases and their control. In *Proceedings of the Fourth Symposium of the International Society for Tropical Root Crops*. IDRC, Ottawa, ON, CA.

Lozano, J. C., Bellotti, A. C., & Vargas, O. (1986). *Sanitary problems in the production of cassava planting material.* In : Proceedings of a global workshop on Root and Tuber Crops Propagation. Pg. 73-85.

Lupski, J. R., & Weinstock, G. M. (1992). Short, interspersed repetitive DNA sequences in prokaryotic genomes. *Journal of Bacteriology, 174(14)*, 4525.

Ma, X. L., Kong, P., You, M. P., Li, H., Sivasithamparam, K., & Barbetti, M. J. (2009). Molecular variation among isolates belonging to eight races of Phytophthora clandestina. *Australasian Plant Pathology, 38*, 608–16.

Martin, B., Humbert, O., Camara, M., Guenzi, E., Walker, J., Mitchell, T., Andrew, P., Prudhomme, M., Alloing, G., Hakenberck, R., Morrison, D. A., Boulnois, G. J. & Claverys, J. P. *(*1992). A highly conserved repeated DNA element located in the

chromosome of *Streptococcus pneumoniae. Nucleic Acids Research, 20,* 3479–83.

McDonald, B. A., & Linde, C. (2002). The population genetics of plant pathogens and breeding strategies for durable resistance. *Euphytica, 124*, 163–80.

McGuire, R. G., & Jones, J. B. (1987). Recovery of *Xanthomonas campestris* pv. *vesicatoria* from Tomato Seed. Plant In: *Pathogenic Bacteria, Current Plant Science and Biotechnology in Agriculture*, Springer, 768.

Mehta, Y. R., Bomfeti, C., & Bolognini, V. (2005). A semi-selective agar medium to detect the presence of Xanthomonas axonopodis pv. malvacearum in naturally infected cotton seed. *Fitopatologia Brasileira, 30(5),* 489-496.

Miesfeld, R. L. (1999). Rapid amplification of DNA. In Applied molecular genetics. New York: A John Wiley & Sons, INC., Publication.

Mkumbira, J. (2002). *Cassava development for small-scale farmers: approaches to breeding in Malawi*. Uppsala: Sweden.

Mortensen, C. N. (2005). *Seed Bacteriology Laboratory Guide*. Danish Seed Health Centre for Developing Countries, Copenhagen, Denmark. Pg.44.

Nassar, A. (1994). Development and selection of apomixis in cassava. *Canadian Journal of Plant Science, 74(2),* 857-858.

Nassar, A. (2006). Cassava genetic resources: extinct everywhere in Brazil. *Genetic Resources and Crop Evolution, 53(7),* 975-983.

Nassar, N. M. (2002). Cassava, Manihot esculenta Crantz, genetic resources: origin of the crop, its evolution and relationships with wild relatives. *Genetics and Molecular Research, 1(4),* 298-305.

Nassar, A. (2007). Wild and indigenous cassava (*Manihot esculenta* Crantz) diversity: An untapped genetic resource. *Genetic Resource and Crop Evolution, 54(3)*, 1523-1530.

Nguyen, T., & Kwoh, C. (2015). Efficient agglomerative hierarchical clustering for biological sequence analysis. In : TENCON 2015-2015 *IEEE Region 10 conference. Pg. 1-3.*

Ntawuruhunga, P., Ssemakula, G., Ojulong, H., Bua, A., Ragama, P., Kanobe, C., & Whyte, J. (2006). Evaluation of advanced cassava genotypes in Uganda. *African Crop Science Journal*, *14(1)*, I-7.

Nweke, F. I. (2004). *New challenges in the cassava transformation in Nigeria and Ghana* (Vol. *118*). International Food Policy Research Institute.

Nweke, F. I. (1994). Processing potential for cassava production growth in Africa.

Ogunjobi, A. A., & Dixon, (2006). Molecular variation in population structure of *Xanthomonas axonopodis pv manihotis* in the south eastern Nigeria. *African Journal of Biotechnology, 5(20)*.

Ogunjobi, A. A., Fagade, O. E., & Dixon, A. G. O. (2008). Physiological studies on *Xanthomonas axonopodis pv. manihotis* (*Xam*) strains isolated in Nigeria. *Advances in Biological Research*, *2(5-6)*, 90-96.

Ogunjobi, A. A., Fagade, O. E., & Dixon, A. G. O. (2010). Comparative analysis of genetic variation among *Xanthomonas axonopodis pv manihotis* isolated from the western states of Nigeria using RAPD and AFLP. *Indian Journal of Microbiology*, *50(2)*, 132-138.

Olive, D. M., & Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology*, *37(6)*, 1661-1669.

Onwueme, C. (1978). The Tropical Tuber Crops: Yam, Cassava, Sweet potato, Cocoyam. *New*

York: John Wiley and Sons Limited.

Onyeka, T. J., Owolade, O. F., Ogunjobi, A. A., Dixon, A. G. O., Okechukwu, R., & Bamkefa, B. (2008). Prevalence and severity of bacterial blight and anthracnose diseases of cassava in different agro-ecological zones of Nigeria. *African Journal of Agricultural Research, 3(4),* 297-304.

Otim-Nape, G. W., & Sengooba, T. (1981). *Cassava bacterial blight disease in Uganda*. In: *Tropical root cropsresearch strategies for the 1980s.* Proceedings of the First Triennial Root Crops Symposium of the International Society for Tropical Root Crops-Africa Branch. September 8-12. Ibadan, Nigeria.

Owiti, J. A. (2009). Molecular and biochemical understanding of post-harvest physiological deterioration in cassava roots and approaches for its modulation.

Owolade, F., Dixon, O., & Adeoti, A. (2006). Diallel analysis of cassava genotypes to anthracnose disease.*World Journal of Agricultural Sciences, 2(9),* 98-104.

Padmaja, G., George, M., Moorthy, S. N., Bainbridge, Z., Plumb, V., Wood, J. F., & Powell, C. J. (1994). Nutritional evaluation of the starchy flour obtained from cassava tubers on fermentation with a mixed-culture inoculum. *Journal of Agricultural and Food Chemistry, 42(3),* 766-770.

Patton, T. G., Katz, S., Sobieski, R. J., & Crupper, S. S. (2001). Genotyping of clinical Serratia marcescens isolates: a comparison of PCR-based methods. *FEMS Microbiology Letters, 194(1),* 19-25.

Restrepo, S., Duque, M., Tohme, J., & Verdier, V. (1999a). AFLP fingerprinting: an efficient technique for detecting genetic variation of *Xanthomonas axonopodis pv. Manihotis*. *Microbiology 145*, 107-114.

Restrepo, S., Duque, M. C., Fregene, M., Bonierbale, M., & Verdier, V. (1999). AFLP assessment of genetic variability in cassava accessions (*Manihot esculenta*) resistant and susceptible to the cassava bacterial blight (CBB). *Genome, 42(2)*, 163-172.

Restrepo, S. (1999). *Etude de la structure des populations de Xanthomonas axonopodis pv. manihotis en Colombie*. Doctoral dissertation. University of Paris VI, France.

Restrepo, S., & Verdier, V. (1997). Geographical Differentiation of the Population of *Xanthomonas axonopodis pv. manihotis* in Colombia. *Applied and Environmental Microbiology, 63(11)*, 4427-4434.

Restrepo, S., Duque, M. C., & Verdier, V. (2000a). Characterization of pathotypes among isolates of *Xanthomonas axonopodis pv. manihotis* in Colombia. *Plant Pathology, 49(6)*, 680-687.

Reynolds, T. W., Waddington, S. R., Anderson, C. L., Chew, A., True, Z., & Cullen, A. (2015). Environmental impacts and constraints associated with the production of major food crops in Sub-Saharan Africa and South Asia. *Food Security*, *7(4)*, 795-822.

Roca, W. M. (1984). Cassava. In W. R. Sharp, D. A. Evans and P.V. Ammirato. *Handbook of plant cell culture, Volume 2, Crop species. New York: Macmillan Publishing Company*, pg. 269-301.

Rodriguez, S., Stapleton, J. J., & Civerolo, E. L. (1987). *Xanthomonas campestris* Involved in Mexican Lime Bacteriosis in Colima, Mexico. In: *Pathogenic Bacteria, Current Plant Science and Biotechnology in Agriculture*, Springer, 658-662.

Rogers, D. J., & Appan, S. G. (1973). *Manihot* and *Manihotoides* (*Euphorbiaceae*), a computer-assisted study. *Flora Neotropica, Monograph no*. 13.

Sander, A., Ruess, M., Bereswill, S., Schuppler, M., & Steinbrueckner, B. (1998). Comparison of Different DNA Fingerprinting Techniques for Molecular Typing of Bartonella henselaeIsolates. *Journal of Clinical Microbiology, 36(10)*, 2973-2981.

Snelling, A. M., Gerner-Smidt, P., Hawkey, P. M., Heritage, J., Parnell, P., Porter, C., & Inglis, T. (1996). Validation of use of whole-cell repetitive extragenic palindromic sequence-based PCR (REP-PCR) for typing strains belonging to the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex and application of the method to the investigation of a hospital outbreak. *Journal of Clinical Microbiology, 34(5)*, 1193-1202.

Terry, E. R., & Oyekan, J. O. (1976). Cassava diseases in Africa reviewed. In *Span*.

Thaveechai, N., Leksomboon, C., Kositratana, W., Paradornuwat, A., & Rojanaridpiched, C. (1993). Survival of *Xanthomonas campestris pv. manihotis* under natural field conditions. *Kasetsart Journal, Natural Sciences*, *27*(1), 25-32.

Trujillo, C. A., Ochoa, J. C., Mideros, M. F., Restrepo, S., López, C., & Bernal, A. (2014). A complex population structure of the Cassava Pathogen *Xanthomonas axonopodis pv. manihotis* in recent years in the Caribbean Region of Colombia. *Microbial Ecology, 68(1)*, 155-167.

UNCTAD, W. (2009). World investment report 2009. *Retrieved February*, *18*, 2010.

Vauterin, L., Hoste, B., Kersters, K., & Swings, J. (1995). Reclassification of *xanthomonas*. *International Journal of Systematic and Evolutionary Microbiology*, *45(3)*, 472-489.

Verdier, V., Berthier, Y., Dongo, P., Chevrier, D., & Boher, B. (1994a). Molecular epidemiology of *Xanthomonas campestris pv. manihotis* causal agent of cassava bacterial blight. *Africa, 1994*, 709–713.

Verdier, V., Boher, B., Maraite, H., & Geiger, J. P. (1994b). Pathological and molecular characterization of *Xanthomonas campestris* strains causing diseases of cassava (*Manihot esculenta*). *Applied and Environmental Microbiology*, *60(12)*, 4478-4486.

Verdier, V., Dongo, P., & Boher, B. (1993). Assessment of genetic diversity among strains of *Xanthomonas campestris pv. manihotis*. *Microbiology*, *139(11)*, 2591-2601.

Verdier, V., Mosquera, G., & Assigbétsé, K. (1998). Detection of the cassava bacterial blight pathogen, *Xanthomonas axonopodis pv. manihotis*, by polymerase chain reaction. *Plant Disease*, *82(1)*, 79-83.

Verdier, V., Restrepo, S., Mosquera, G., Jorge, V., & Lopez, C. (2004). Recent progress in the characterization of molecular determinants in the *Xanthomonas axonopodis pv. manihotis*–cassava interaction. *Plant Molecular Biology*, *56(4)*, 573-584.

Versalovic, J., Koeuth, T., & Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research*, *19(24)*, 6823-6831.

Versalovic, J., Schneider, M., De Bruijn, F. J., & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, *5(1)*, 25-40.

Willems, A., Gillis, M., Kersters, K., Van Den Broecke, L., & De Ley, J. (1987). Transfer of *Xanthomonas ampelina* Panagopoulos 1969 to a New Genus, Xylophilus gen. nov., as *Xylophilus ampelinus* (Panagopoulos 1969) comb. nov. *Internal Journal of Systemic Bacteriology*, 1987; *37(4)*: 422-430.

Wydra, K. (2002).The concept of resistance, tolerance and latency in bacterial diseases: examples from cassava and cowpea. In: *Ninth Symposium on 'New Aspects of Resistance Research*

on Cultivated Plants,'Bacterial Diseases. Aschersleben, Germany. Beiträge für Züchtungsforschung BAZ 8 (3), 36-43.

Wydra, K., & Verdier, V. (2002). Occurrence of cassava diseases in relation to environmental, agronomic and plant characteristics. *Agriculture, Ecosystems and Environment, 93(1),* 211-226.

APPENDIX

Appendix 1: Table of numerical scores of morphological colony characteristics of Xam isolates

Phenotypic characteristic	Distinctive	Score
Form	Circular	1
	Fusiform	2
	Rhizoid	3
	Irregular	4
	Filamentous	5
Color	White	1
	pale yellow	2
	Gray	3
	Brown	4
	Green	5
	Cream	6
Elevation	Convex	1
	Concave	2
	Raised	3
	Umbonate	4
	Domed	5
	Flat	6
Size	Medium	1
	Small	2
	Large	3
Surface	Smooth	1
	Wavy	2
	Rough	3
	Granular	4
	Pappilate	5
Edges	Entire	1
	Undulate	2
	Crenated	3
	Fimbriate	4
	Curled	5
Structure	Opaque	1
	Translucent	2
	Transparent	3
Degree of growth	less growth	1
	moderate growth	2
	high growth	3
	very high growth	4
	no growth	5

Texture	Dry	1
	Moist	2
	Mucoid	3
	Brittle	4
	Viscous	5
	Butyrous (buttery)	6